NOVEL NUTRITIONAL AND BIOTECHNOLOGICAL APPROACHES TO CONVERT DEFATTED MICROALGAL BIOMASS AND POULTRY FEATHERS INTO HIGHLY SUSTAINABLE FEED PROTEINS

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ABSTRACT

With our global population on the rise, there is a need not only for alternative sources of energy for both humans and animals, but also for alternative food sources to maintain food security. Microalgae from biofuel production and poultry feather waste are both rich in protein, and stand as promising candidates for supplementation into animal diets as protein-rich feed additives. In doing so, the corn and soybean meal that traditionally make up the majority of an agricultural animal's diet, may be re-allocated for human consumption. Additionally, developing new uses for a waste product of the biofuel industry (microalgae) and poultry industry (feathers) would help alleviate environmental pollution.

We aimed to 1) determine the nutritional potential of supplementing microalgae biomass into the diets of weanling pigs, without negatively affecting their growth performance or health, and 2) improve the digestibility of poultry feathers *in vivo* through the use of cloned and expressed microbial enzymes in *in vitro* feather hydrolysis.

Protein-rich microalgal biomass from biofuel production stands as a promising new animal feed source, and may serve as an alternative to corn and soybean meal (SBM) in animal diets. Our objective was to determine potential and limitation of a new diatom microalgal species *Staurosira sp* (full-fat microalgae) and its defatted algal biomass generated from biofuel production in replacing SBM and corn in diets for weanling pigs. Two experiments were conducted. In the first experiment pigs were fed a corn and SBM basal diet (BD), BD + 7.5% de-fatted algae replacing SBM, and BD + 7.5% or 15% de-fatted algae replacing corn and SBM for

6 wk. We found that the inclusion of algae in the diet to replace a significant portion of SBM lowered the average daily gain and feed efficiency of the pigs. Feeding pigs 15% defatted algae resulted in lower fecal microminerals Cu, Se, and Zn. The health status of the pigs, as determined by plasma biochemical assays, was not negatively affected. It was hypothesized that either the micromineral deficiencies, or the acid:base balance of the algae biomass negatively affected the growth performance of the pigs. A second study was conducted on the full-fat algae with and without the inclusions of either fumaric acid or the minerals found to be limited in Experiment 1. The inclusion of fumaric acid with algae alleviated the growth losses of algae fed to pigs alone. It was feasible to supplement 7.5% de-fatted algae or 10% full-fat with an organic acid for a replacement of the same amount of corn and SBM in diets for weanling pigs, without adverse effect on growth performance, biochemical status, or fecal excreta.

Previous research on hydrolyzing keratinous feathers suggests that microbial proteases and reductases may be key players in their effective degradation. We describe the first heterologous protein expression of 3 predicted serine proteases and 7 predicted thioredoxin disulfide reductases from *Streptomyces fradiae* var. k11. The *in vitro* feather hydrolysis assay showed that the inclusion of reductases and proteases, with the additions of a known chemical reducing agent (β-mercaptoethanol) and protease (Proteinase K), apply a synergistic effect in feather keratinolysis. As such, these cloned and expressed enzymes have promising potential for industrial uses and feather keratin.

BIOGRAPHICAL SKETCH

Krystal Lum was born on April 26th, 1989, and raised in Queens, New York, to Kwok and Mimi Lum. She has two older brothers, Eric and Kevin. She attended Townsend Harris High School in Flushing, New York, where she developed an appreciation for the humanities and science research. In 2007, Krystal spent her freshman year of undergrad at Tufts University in Medford, Massachusetts, majoring in biochemistry. She transferred a year later to Cornell University as an animal science major, and first joined the lab of Dr. Xingen Lei in Fall 2009 as a Mouse Room Attendant. Krystal subsequently graduated in 2011 with distinction in research, and pursued a Master in Science with Dr. Lei. Upon completing this degree, Krystal will join the Department of Molecular Biology at Princeton University for her Ph.D in Fall, 2013.

To my loving parents

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7.5% DFA-A	Swine diet with 7.5% defatted algae replacing soybean meal
7.5% DFA-B	Swine diet with 7.5% defatted algae replacing soybean meal and corn, in Experiment 1
15% DFA	Swine diet with 15% defatted algae replacing soybean meal and corn, in Experiment 1
10% FFA	Swine diet with 10% full fat algal biomass replacing soybean meal and corn, in Experiment 2
10% FFA + FA	Swine diet with 10% full fat algal biomass replacing soybean meal and corn, and addition of 2% fumaric acid, in Experiment 2
10% FFA + TM	Swine diet with 10% full fat algal biomass replacing soybean meal and corn, and addition of trace minerals Cu, Se, and Zn, in Experiment 2
ADFI	Average daily feed intake
ADG	Average daily gain
BCA	Bicinchoninic acid
BD	Basal diet
BD1	Basal diet in Experiment 1
BD2	Basal diet in Experiment 2
BW	Body weight
DFA	Defatted microalgal biomass
DHA	Docohexaenoic acid
FI	Feed intake
FFA	Full fat microalgal biomass
G:F	(Weight) Gain to feed ratio

MEROPS	Database of proteolytic enzymes, their substrates, and inhibitors (No acronym definition available)
MS	Mass spectrometry
P1, P2, P3	Serine protease
RAST	Rapid Annotation Subsystem Technology
SBM	Soybean meal
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
TrxR	Thioredoxin disulfide reductase
WT	Wild type

CHAPTER ONE

Introduction

1.1 The need for alternative energy and protein sources for humans and animals

Today's global population is in excess of 7 billion people; its projection to climb to over 9 billion within the next several decades (1, 2) garners a revolutionary demand for alternative renewable energy and food sources. This population growth presents a serious challenge to managing food security for both humans and animals. Corn and soybean meal are staple food crops for humans, and are also the conventional feedstuffs that provide nutritional energy and protein in agricultural animal diets. Quantitatively, protein is the single most expensive nutrient in animal diets and is essential in maintaining proper health status and physiological function (3, 4). Considering the rise in worldwide population, the extensive incorporation of corn and soy in animal diets directly competes with their availability for human consumption. This allocation of food is largely unsustainable, and alternatives to corn and soybean meal in animal diets is necessary to both establish and maintain a more harmonious infrastructure between human needs and animal agriculture.

1.2 Impacts of animal agriculture on the environment

To meet the food demands of over 7,000 million people, animal production systems in modern agriculture have been intensified. Rich nutrient sources are supplemented into animal diets, while the diet ingredients themselves are grown on arable land supplemented with synthetic fertilizers, both of which impose a substantial degree of environmental liability (5).

Oftentimes, intensive animal agriculture generates an excess of nutrients that pollutes the surrounding habitats and ecosystems in the form of phosphorus and nitrogen from manure (6). In the United States alone, a minimum of 133 million tonnes of manure is generated annually (7), entering the surrounding atmosphere, water, and soil by means of volatilization, runoff, and leaching (5). Animal agriculture contributes to 18% of all anthropogenic greenhouse gas emissions, and 65% of all anthropogenic nitrogen released into the atmosphere (8), causing acidification of non-agricultural eco-systems and the development of acid rain (9). Nitrogen runoff that enters either coastal or fresh waters results in eutrophication that significantly diminishes the quantity and diversity of sea life (10). The challenge arises to improve the sustainability of animal agriculture, while intensifying its practices to feed an imminent 9,000 million people and diminish food competition between humans and animals, without exacerbating its contribution to environmental pollution. Two such alternatives may be replacing corn and soybean meal in animal diets with marine microalgae from biofuel production, and feathers from poultry production, both of which present a potential means to achieve food security without compromising environmental integrity.

1.3 Marine microalgae has the potential to be an effective, eco-friendly feed additive

As global supplies of petroleum continue to decline, renewable natural fuels are being targeted to resolve the search for alternative energy sources. Biofuel, or energy derived from biological raw materials, shows promise in harnessing adequate energy and reducing greenhouse gas emissions by replacing exhaustive fossil fuels. Marine microalgae cells themselves have generated interest not only for their ability to sequester carbon dioxide (CO_2), but also for their

rapid photosynthetic conversion of CO_2 into reusable biomass such as methane with maximum capture or hydrogen (11, 12). The incorporation of microalgal biomass into animal feeds is well matched to alleviate the food allocation imbalance between humans and animals, as well as provide an alternative energy source in the form of biodiesel. Microalgal leftovers derived from prior cultivation for biofuel presents itself as a promising carbon-neutral animal feed supplement (13-15).

1.3.1 Biofuel production from algae

Since the 1940's, it was already determined that microalgae were capable of yielding high amounts of cellular lipids and metabolites through selective growth pressures. Algae was not viewed as a potential energy source until the 1950's, and was initially tested for methane gas production by the anaerobic digestion of their cell carbohydrates (16, 17). To cultivate microalgae for eventual harvesting and processing resulting in biodiesel and co-products, either photoautotrophic or heterotrophic methods may be implemented. In a photoautotrophic system, microalgae are grown in the presence of light within constructs such as open raceway ponds. Heterotrophic approaches, whereby algae are grown in darkness and supplemented with selected carbon sources, commonly include closed photobioreactors or fermentation tanks. Depending upon the optimal growth conditions of the microalgal species in use, one or more systems may be preferred over others. Algae cultivation setups largely aim to preserve limited arable land for conventional farming. De-watering and lipid extraction of the cultivated algae, by both physical and chemical means, subsequently allow algal metabolites and biomass to be separated and converted for use as fuel precursors and co-products (18). As a co-product of biofuel production,

the leftover defatted microalgal biomass may be used as animal feed. While experimentation on the viability of algae cultivation, harvesting, and conversion has proven successful, algae's large-scale cultivation, processing, and refinement under feasible commercial parameters has yet to be fully explored.

1.3.2 Algae in animal nutrition

Microalgae has long been studied as an unconventional feedstock for animal diets, dating as far back as the 1950's. Their successful incorporation in animal feed has been dependent upon effective algal digestibility under biological conditions including digestion time, pH, temperature, and changes in health status. Processes to culture algae in ponds were first determined, quickly followed by supplementation of algae into animal diets as a protein source (19, 20). The discovery that different sources of cultivated algae were successful in maintaining animal growth parameters and, in some cases, improving growth was joined by studies on the specific nutrient contents of algae. In 1957, 10% sewage-grown algae that was high in carotenoids and contained at least 40% crude protein was supplemented as a viable protein source for chicks (21). During the following decade, pond and tap water-grown algae were found to sustain fish growth, and growing-finishing pigs fed a barley diet with 6-10% sewage-grown algae maintained growth rate and feed conversion efficiency (22, 23). The progress made in researching the effects of algal supplementation in agricultural animals has opened a biomedical gateway for improving common micronutrient deficiencies in humans. A study conducted in the last decade, on pigs fed algae consisting of naturally high iodine, showed a 10% elevation in daily body weight gain, allowing for marketability studies in producing iodine-rich meat for human consumption (24).

1.3.3 Nutrient profile of protein-rich microalgae in animal agriculture

As such, the increased regard for algae as a food source came with discoveries of the relatively nutrient-rich profile of many algal species. While the nutritional profile of microalgae varies considerably with the species at hand, a large majority are characterized by protein, carbohydrate, and lipid values comparable, if not superior, to traditional feedstuffs (Table 1.1) (25-27). Dietary soybean meal typically contains up to 48% crude protein, and lays claim to a relatively well balanced amino acid profile. The diverse nutrient profiles make different algae species amenable to cultivation for diet-specific needs in both human and animal nutrition. A commonly cultivated algae for human consumption, *Spirulina maxima*, also contains advantageous values, including high levels of vitamin B_1, B_2, β-carotene, and up to 71% crude protein, consisting of all essential amino acids at sufficient concentrations, with the exception of minor inadequacies in those containing sulfur, a common deficiency in many algal species (Table 1.2) (28, 29). Because protein is considered to be the most expensive nutrient included in swine diets (3), developing natural alternatives to corn and soybean meal may be extremely cost effective. After biofuels are extracted from cultivated microalgae, the resultant biomass leftover shows feasibility in replacing traditional protein sources in the diets of both monogastric and ruminant animals.

A previous study supplementing lipid-extracted microalgal biomass in the diets of weanling pigs show that the high protein content alone was capable of partially replacing 7.5% corn and soybean meal without affecting overall growth performance and plasma biochemical indicators of health (30). Similarly in laying hens, a 7.5% replacement of corn and soybean meal with defatted algal biomass did not negatively affect hen production parameters or health, yet significantly increased the redness of the yolk, and decreased both the lightness and yellowness

Table 1.1 Proximate nutrient composition of conventional feedstuffs and various whole algae (% dry matter). Adapted from (15, 25-27).

Source	Crude Protein	Carbohydrates	Lipids
Soybean	37	30	20
Corn	10	85	4
Wheat	14	84	2
Anabaena cylindrical	43-56	25-30	4-7
Arthrospira maxima	60-71	13-16	6-7
Chlorella vulgaris	51-58	12-17	14-22
Spirogyra sp.	6-20	33-64	11-21
Synechococcus sp.	73	15	11

Table 1.2 Amino acid profile of conventional protein sources and various whole algae (g/100 protein) (15).

Source	Ala	Arg	Asp	Cys	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Try	Tyr	Val
Egg	-	6.2	11.0	2.3	12.6	4.2	2.4	6.6	8.8	5.3	3.2	5.8	4.2	6.9	5.0	1.7	4.2	7.2
Soybean	5.0	7.4	1.3	1.9	19.0	4.5	2.6	5.3	7.7	6.4	1.3	5.0	5.3	5.8	4.0	1.4	3.7	5.3
C. vulgaris	9.4	6.9	9.3	-	13.7	6.3	2.0	3.2	9.5	6.4	1.3	5.5	5.0	5.8	5.3	-	2.8	7.0
D. bardawil	7.3	7.3	10.4	1.2	12.7	5.5	1.8	4.2	11.0	7.0	2.3	5.8	3.3	4.6	5.4	0.7	3.7	5.8
S. platensis	9.5	7.3	11.8	0.9	10.3	5.7	2.2	6.7	9.8	4.8	2.5	5.3	4.2	5.1	6.2	0.3	5.3	7.1
Aphanizomenon flos-aquae	4.7	3.8	4.7	0.2	7.8	2.9	0.9	2.9	5.2	3.2	0.7	2.5	2.9	2.9	3.3	0.7	-	3.2

of the egg yolks from algae-fed hens (31). However, its inclusion at over 10% reduced feed intake, and affected protein metabolism by reducing plasma uric acid and egg albumen weight. Up to a third of soybean meal was successfully replaced in the diets of weanling pigs by algae biomass from the cyanobacteria *S. maxima*, *Arthrospira platensis*, and *Chlorella* sp (32). In a study conducted on laying hens, a maximum inclusion of 10% *Porphyridium* sp. red algal biomass was supplemented into the diet without affecting overall body weight, egg count, or egg weight, and further lowered cholesterol levels by 24% (33).

In spite of the successful algal inclusion rates in animal diets, limiting factors associated with the proteinaceous biomass may be the presence of non-protein nitrogen in the crude protein analyses, which consists of nucleic acids, nitrogen-containing cell walls, and amines. As single cell proteins, microalgae are photosynthetic unicellular eukaryotes, which contain nucleic acids. These nucleic acid values may thus be included in the crude protein amount, at rates of approximately 10% (29). In this way, the viability of precise algal feedstuff replacement in the diet, on the basis of crude protein, may be hindered by the inclusion of non-protein nitrogen.

To date, little research has been conducted on approaches to improve the digestibility of algae. Of notable concern is the algal cell wall, which is indigestible by monogastric animals, yet comprises approximately 10% cellular dry matter (34). Approaches to alleviate this challenge include additional enzymatic treatments to enhance cell wall and algal digestibility (34), as well as the generation of genetically mutant algae that lack cell walls (35). A green algae *Chlamydomonas reinhardi* was treated with a chemical mutagen that resulted in defective cell walls, as evidenced by their changes in colony morphology. Of those mutants showing a complete absence of cell walls, the amino acid composition was shown to be largely comparable to its native, cell well-containing form. As such, it is possible to exploit cell wall-lacking, yet

isolatable, mutant algae for commercial cultivation and both biofuel and animal agricultural uses. Algal cell solubility within biological conditions may pose an additional problem to its digestibility, since previous research indicates algal solubility is greatest under alkaline parameters (36).

Beneficially, microalgae may be cultivated for their advantageous fatty acid profile (Table 1.3), with notable enrichment in essential polyunsaturated fatty acids, including arachidonic acid, docohexaenoic acid (DHA), eicosapentaenoic acid, and γ–linoleic acid (18, 37). Essential fatty acids are incapable of being biologically produced by either humans or animals. In this way, the rich synthesis of essential fatty acids by a variety of algal species represents a largely untapped natural resource with multiple health implications, not only as a directly extractable health food for humans, but also as an algal supplement for agricultural animals. In the case of supplementing essential fatty acid-rich algal biomass to laying hens, it may be possible to improve the nutritional value of eggs. The production of DHA from algal biomass alone is a rapidly growing market last estimated at less than 300 tons produced per year, yet generating $1.5 billion each year (38, 39).

Table 1.3 Generalized fatty acid profiles of oil extracts from *Spirulina maxima* (SP), *Chlorella vulgaris* (Cv), *Scenedesmus obliquus* (Sc), *Dunaliella tertiolecta* (Dt), *Nannochloropsis* sp, (Nanno), and *Neochloris oleabundans* (Neo) (37).

Fatty Acid[1]	Sp	Cv	Sc	Dt	Nanno	Neo
14:0	0.34	3.07	1.48	0.47	7.16	0.43
16:0	40.16	25.07	21.78	17.70	23.35	19.35
16:1	9.19	5.25	5.95	0.88	26.87	1.85
16:2	N.D.	N.D.	3.96	3.03	0.39	1.74
16:3	0.42	1.27	0.68	1.24	0.48	0.96
16:4	0.16	4.06	0.43	10.56	N.D.	7.24
18:0	1.18	0.63	0.45	N.D.	0.45	0.98
18:1	5.43	12.64	17.93	4.87	13.20	20.29
18:2	17.89	7.19	21.74	12.37	1.21	12.99
18:3	18.32	19.05	3.76	30.19	N.D.	17.43
18:4	0.08	N.D.	0.21	N.D.	N.D.	2.10
20:0	0.06	0.09	N.D.	N.D.	N.D.	N.D.
20:1	N.D.	0.93	N.D.	N.D.	N.D.	N.D.
20:2	0.48	N.D.	N.D.	N.D.	N.D.	N.D.
20:3	N.D.	0.83	N.D.	N.D.	N.D.	N.D.
20:4	N.D.	0.23	N.D.	N.D.	2.74	N.D.
20:5	N.D.	0.46	N.D.	N.D.	14.31	N.D.
Saturated	41.74	28.86	23.71	18.17	30.96	20.76
Unsaturated	14.62	18.82	23.88	5.75	40.07	22.14

[1]The number preceeding the colon represents the number of carbons in the fatty acid chain. The number proceeding the colon represents the number of double bonds in the chain.

1.3.4 Challenges to implementing microalgae in animal agriculture

Recent estimates indicate that 30% of global algal production was marketed for applications in the animal feed industry (29), amounting to a fast-growing $300 million in retail value (38). Despite the encouraging feasibility of incorporating microalgal biomass into animal feed, its replacement of either soybean meal or corn is nowhere near cost-effective due to its extensive processing to extract biofuel resources. Species-specific large-scale algae refinery techniques must be placed at the forefront of focus in order to reduce the cost of production for marketable use as an animal feedstuff. Use of algal biomass in animal feed not only alleviates human and animal food security, but also contributes to reductions in environmental pollution, promotes more sustainable animal agriculture, as well as generates alternative energy in the form of biofuel. As such, it is important to support and continue research efforts to elucidate the limiting factors behind full algal digestion *in vivo*, determine mechanisms by which digestion and nutrient utilization may be improved, and discover nutritionally advantageous strains for large-scale cultivation. Extensive research on the long-term supplementation effects of algae in animal diets and bioactive compounds have yet to be conducted.

1.4 Poultry feather waste has the potential to be a more bioavailable protein-rich feed additive

Approximately five million tons of chicken feathers accumulate annually (40), estimated from a global consumption of over 86 million tons of chicken meat (41). Feathers are a significant by-product of the poultry industry largely deposed in landfills, and thus contribute to

environmental harm in the form of nitrogen pollution (42). While a by-product, feathers are surprisingly rich in protein (Table 1.4) (43), comprised of up to 90% keratin primarily as β-keratin (44). Its environmentally undesired ubiquity and proteinaceous character lends itself as an agreeable and valuable raw material to generate a nutrient-dense, eco-friendly, cheap animal feed additive (45, 46). In accordance with its keratinous structure, indeed, proximate analysis of hydrolyzed feather meal shows high levels of crude protein (Table 1.5). However, its molecularly sound structure makes it recalcitrant and resistant to effective proteolytic digestion *in vivo* by enzymes such as pepsin and trypsin (47). Feathers are tightly packed polypeptide chains of supercoiled a-helices and b-sheets. These interwoven chains are further crosslinked by a high degree of disulfide bonds, as cysteine bridges. The presence of extensive hydrogen bonding enhances a stable hydrophobic interaction, all of which promotes the strength of feather's rigid keratin (46).

To produce a more digestible and proteinaceous feed additive, feather waste is hydrothermically treated, a procedure whose thermo-energetically steep cost may not justify its use as a poorly digestible feedstuff (46). Studies have indicated that hydrothermal processing often leads to the destruction of essential amino acids and the formation of non-nutritive amino acids, including lysinoalanine and lanthionine (45, 48, 49). Previous research determined that feather meal supplemented into the diets of chickens is capable of preventing weight loss in comparison to protein-deficient chicks on a limited basis only, owing to the relatively low bioavailability of methionine, lysine, and tryptophan (46, 50, 51). Thus, enhancements in the nutritional value of feather waste would be of significant value to the sustainability of animal agriculture and improvement of food security.

Table 1.4 Feather amino acids for mixed sex broilers at 42 d old (43).

Protein %	95.7%
Amino acid (%):	
Arg	6.8
Cys	7.2
His	0.6
Iso	4.6
Leu	7.9
Lys	1.9
Met	0.7
Phe	4.8
Thr	4.9
Trp	0.7
Tyr	2.6
Val	6.5
Total essential	49.1

Table 1.5 Proximate nutrient composition of hydrolyzed feather meal (% dry matter)[1].

Component	Hydrolyzed feather meal
Crude protein	91.0
Crude fat	8.3
Ash	2.19
Total digestible nutrients	80.0
Calcium	0.42
Phosphorus	0.26
Magnesium	0.05
Potassium	0.12
Sodium	0.20
Sulfur	1.89

[1]Nutrient composition was analyzed by Dairy One, INC. Ithaca, NY 14850.

1.4.1 Microbial hydrolysis of poultry feathers may depend on proteases and disulfide reductases

Concerted efforts to hydrolyze feather waste for improved amino acid balance and digestibility have focused on microbial fermentation for species such as those belonging to *Bacillus*, *Aspergillus*, and *Streptomyces*, which has shown remarkably effective abilities to use keratin as a nutritional substrate (47, 52). Feather lysate from feather fermentation with *Bacillus licheniformis* PWD-1 resulted in a feed protein with a similar protein profile to that of soybean meal (53). While the exact mechanism of hydrolysis is still unknown, it is well-accepted that microbial enzymes aid in modifying the structure of feather keratin, making it more susceptible to effective proteolysis (43, 51). Interestingly, microbial keratinases [EC 3.4.21/24/99.11] have been consistently found to be produced in the presence of keratinous substrates, but purified samples have yet to completely solubilize keratin in its native form (54, 55).

Keratinases are widespread in nature, and include an extensive suite of serine or metallo-proteases, implicated in the proteolysis of feather keratin's extensive peptide bonding (42). It is widely-supported that feather degradation by serine proteases may be due to their preference and ability to cleave hydrophobic and aromatic residues at the P1 site of keratin (56). Because hydrophobic residues pervade the surface of feathers, this idea explains why digestive enzymes like pepsin, whose preference is for hydrophilic lysine and arginine, are incapable of superficial feather degradation; the inner feather structure where peptidyl lysines and arginines are present, are inaccessible (57). The ineffectiveness of keratinases to hydrolyze feather waste individually has led many to additionally focus on the abundance of disulfide bonds in keratin. The release of thiol groups has been discovered during microbial fermentation, supporting the attribution that the reduction of keratin's disulfide bonds is critical to allow site-specific proteolysis to occur (58-60). The inclusion of strong chemical reducing agents such as β-mercaptoethanol, sodium sulfite and dithiothreitol has shown to aid in hydrolysis, yet are too toxic for feed applications. Thus, feather keratinolysis appears to be a cooperation action involving reduction and

proteolysis. Despite the plausibility of serine protease activity requiring assisted redox, the use of proteases and enzymatic reductases both expressed from microbes remains scant in the literature.

1.4.2 Streptomyces sp. capable of degrading feathers contains a thioredoxin disulfide reductase subsystem and expresses serine proteases in the presence of feathers

Previous work in our lab generated a low quality 3.3 Mb draft genome of the feather-degrading bacteria, *Streptomyces fradiae* var. k11, by Illumina paired-end sequencing and annotation with the Rapid Annotation Subsystem Technology (RAST) server. An analysis of subsystems within the genome showed the presence of 8 genes categorized under the subsystem, "Thioredoxin disulfide reductase." An additional experiment was conducted in which a culture of *S. fradiae* var. k11 was induced with feathers, to isolate and identify potential enzymes involved in the keratinolytic degradation. Analysis of the supernatant with a 2D spot gel, tandem MS/MS, and quantitative 2D-LC MS/MS proteomics revealed 59 newly secreted proteins compared to the control un-induced culture, of which 16 were identified as predicted proteases/peptidases, including serine proteases.

1.4.3 Classification of serine proteases

Serine proteases are a family of enzymes that comprise roughly two-fifths of all identified proteases, and have been found in eukaryotes, prokaryotes, archae, and viruses (61). Defined mechanistically by the active site, serine proteases contain a nucleophilic serine (Ser) residue that acts in concert with single aspartic acid (Asp) and histidine (His) residues, in what is coined as the "catalytic triad" (62). On a structural basis, these proteases are categorized into 15 clans by the *MEROPS* peptidase database by similarity in amino acid sequence and homology (Table 1.6). The 4 clan subfamilies containing the most protease members may be epitomized by subtilisin, chymotrypsin, prolyl oligopeptidase, and wheat serine carboxypeptidase II (Figure

1.1) (61). The distinct clans show dissimilar secondary and tertiary structural motifs, yet maintain a conserved geometric orientation of the catalytic serine and histidine residues (63). While not geometrically conserved, the aspartic acid maintains its presence and function within the catalytic triad.

1.4.4 Serine protease mechanism of action

Serine proteases require activation through the cleavage of the precursor pre-proprotein zymogen (64). In the case of chymotrypsin-like serine proteases, cleavage occurs after the 15th residue, which thereafter induces a conformational change that is modulated by an oxyanion hole (65). The oxyanion hole, formed by a positively-charged ion-pair with the NH peptidyl backbone groups of glycine-193 and serine-195, serves to stabilize the tetrahedral transition state that follows Serine's nucleophilic oxygen attack on the carbonyl carbon of the substrate's peptide bond (Figure 1.2). At first the Asp polarizes His, which functions as a base, de-protonating the Ser-195 hydroxyl group. The nucleophilic attack by Ser forms a tetrahedral transition state, which produces a covalent acyl-enzyme intermediate upon the donation of a proton from His to the newly formed amine group (63, 65). A nucleophile water molecule displaced the leaving group of the polypeptide fragment, attacking the acyl-enzyme intermediate and thus forming a second tetrahedral intermediate, and further resulting in a new substrate C-terminus (66).

Table 1.6 Serine protease clans[1], classified on the basis of structural similarities (61).

Peptidase Clan[2]	Total Identifiers	Major Subfamily Type Example
PA	712	Chymotrypsin
PB	42	EGF-like module containing mucin-like hormone receptor-like 2
PC	3	Dipeptidase E
SB	218	Subtilisin
SC	458	Prolyl oligopeptidase
SE	49	D-Ala-D-Ala carboxypeptidase B
SF	40	Signal peptidase I
SH	11	Cytomegalovirus assemblin
SJ	27	Lon-A-peptidase
SK	43	Clp protease
SO	2	*Escherichia coli* phase K1F endosialidase CIMCD self-cleaving protein
SP	11	Nucleoporin 145
SR	11	Lactoferrin
SS	3	Murein tetrapeptidase LD-carboxypeptidase (*Pseudomonas*-type)
ST	40	Rhomboid-1 (Diptera)

[1]The *MEROPS* database classifies peptidases hierarchically by structure, whereby each peptidase is categorized into a family by amino acid sequence similarity, and families are placed into clans based on homology. The clans and identifiers were tabulated from *MEROPS* Release 9.8.

[2]The *MEROPS* database abbreviates clans with the first letter describing the catalytic type of families included, whereby "P" contains more than one catalytic type and "S" stands for Serine. The second letter arbitrarily describes sequential clans.

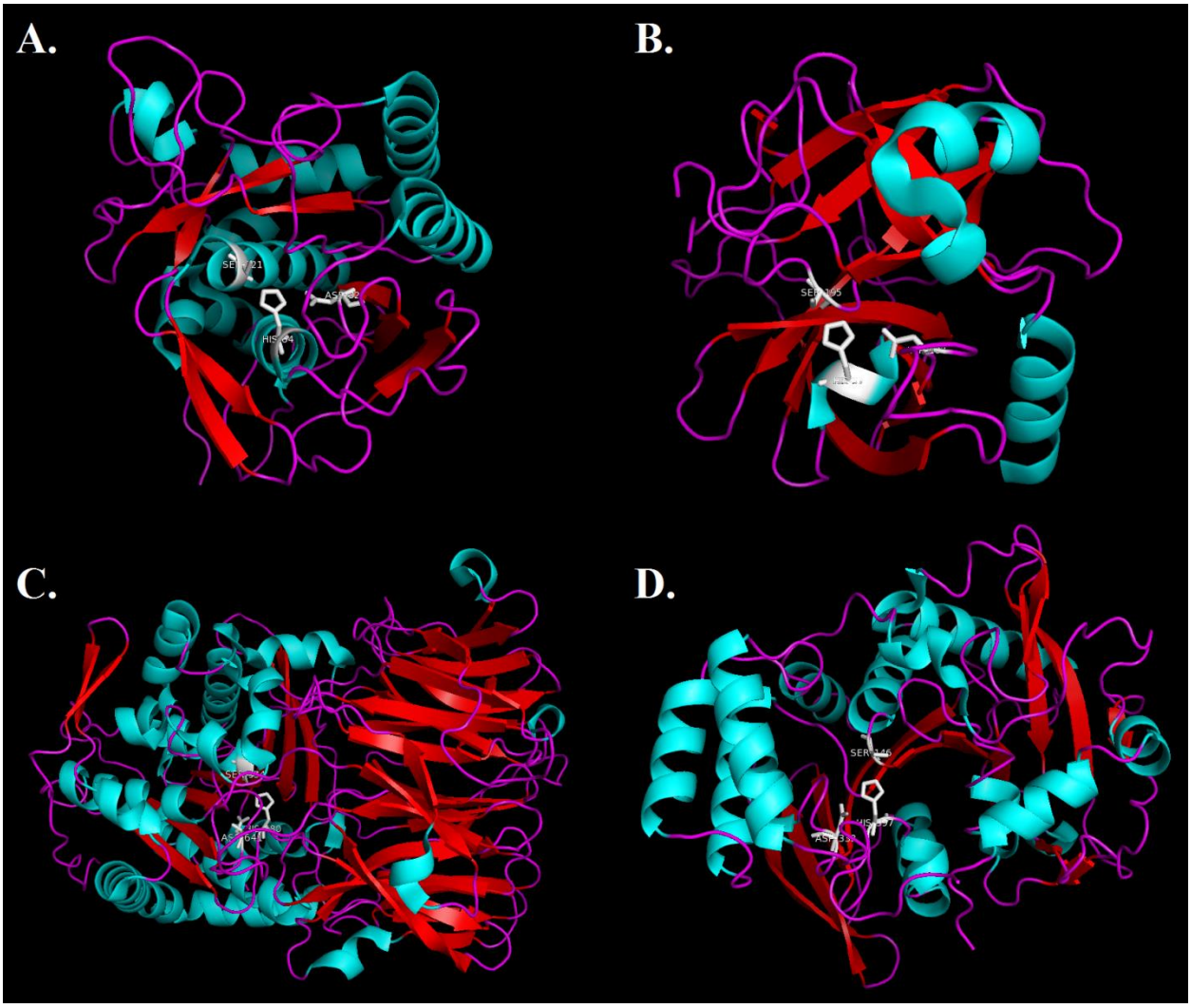


Figure 1.1 Representative structural motifs of the 4 most abundant clans of serine proteases, as typified by (A) subtilisin (PDB file 1ST2), (B) chymotrypsin (PDB file 2P80), (C) prolyl oligopeptidase (PDB file 4BCB), and (D) wheat serine carboxypeptidase II (PDB file 3SC2). Images are colored by secondary structure; α-helices are shown in aqua, β-sheets in red, and loops in purple. The conserved catalytic triads are highlighted and labelled as white sticks. Images were prepared using PyMOL Molecular Graphics System (Portland, OR).

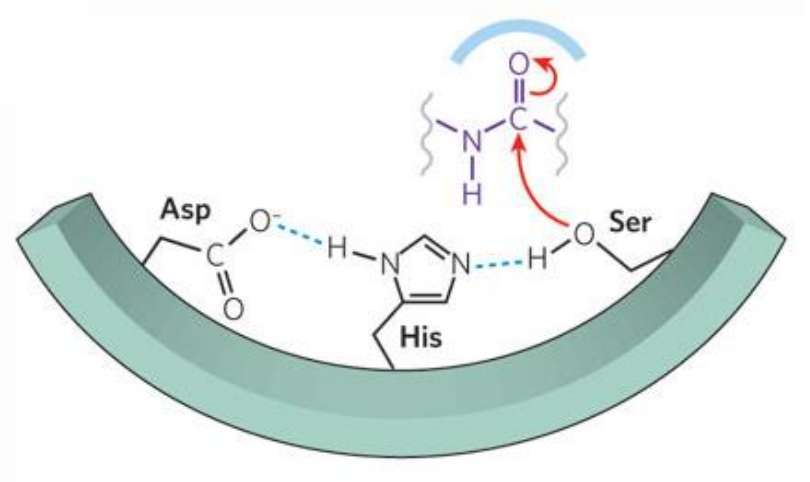


Figure 1.2 Representative mechanism of the serine protease catalytic triad. The green curve is a schematic for the rest of the enzyme. The small blue curve represents the oxyanion hole. The dotted blue lines are hydrogen bonds or electrostatic interactions. The red arrow represents the nucleophilic attack of Ser-195 on the scissile carbonyl carbon of the substrate peptide (65).

1.4.5 Classification of thioredoxin disulfide reductases

Thioredoxin reductases belong to the thioredoxin system comprised of the reductase (TrxR) and its redox active protein, thioredoxin (Trx), categorized under the family of dimeric flavoenzymes that reduce pyridine nucleotides and disulfide and dithiol compounds (67). TrxRs are homodimeric proteins with each monomer containing an FAD prosthetic group domain and NADPH-binding site (Figure 1.3). The catalytic reduction of the Trx substrate is carried out with the incorporation of electron donor NADPH. Trx are known for their ubiquity as an electron source for multiple metabolic processes; their reduction supplies reducing equivalents to several oxidoreductases, such as to ribonucleotide reductase for the reduction of ribonucleotides to deoxyribonucletides during DNA synthesis; to transcription factors, which facilitate binding to DNA and modifying gene transcription; to Trx peroxidase for the conversion of hydrogen peroxide to water (68, 69). As homologues of both proteins are found in nearly all living organisms, the thioredoxin system is well-conserved across kingdoms (70). All TrxR are active as dimers, and in *E. coli* contain 2 redox active cysteines that serve as the catalytic site, Cys-Ala-Thr-Cys (71). In mammalian organisms, cells not only contain machinery for the Trx-TrxR system, but also for the glutathione system (Figure 1.4), which functions similarly as a redox system for thiol/disulfide compounds. Despite the conservation of the Trx-TrxR system from prokaryotes to eukaryotes, the roles of thioredoxin reductase in actinomycetes, including *Streptomyces* sp., are thought to be more complex in fulfilling the reducing roles of both Trx and glutathione. Actinomycetes are believed to have a much more complex redox system than other bacterial species owing to their unique multicellular development, which includes differentiation of the organism into ‘isoforms,’ such as aerial hyphae, filamentous vegetation, and reproductive spores. Thus, complete functional analysis of thioredoxin reductases in bacterial species, namely actinomycetes, have yet to be fully elucidated (72).

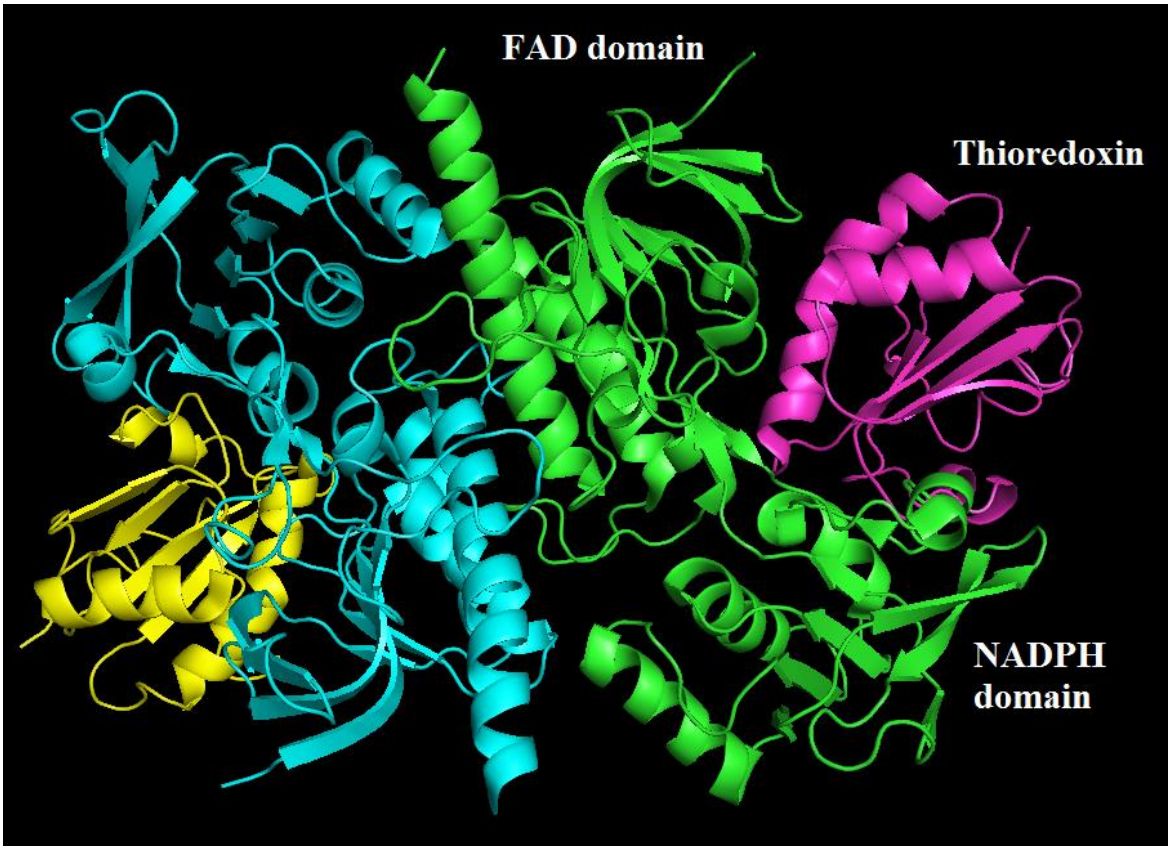


Figure 1.3 Representative structure of 1 monomer within the dimeric thioredoxin reductase in *E. coli* (PDB file 1F6M). A thioredoxin molecule, FAD domain, and NADPH domain are labeled. The image was prepared using PyMOL Molecular Graphics System (Portland, OR).

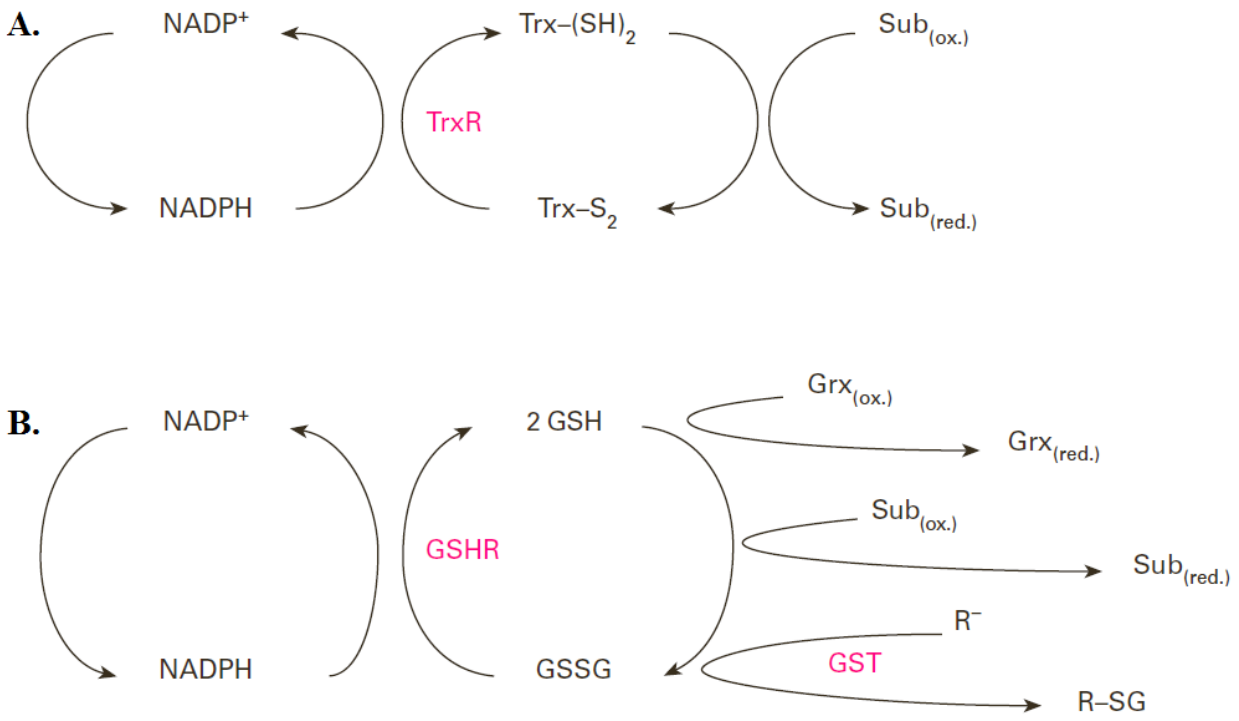


Figure 1.4 Schematic of the (A) TrxR reduction system, catalyzing disulfide bonds in the presence of NADPH. Substrates include Trx, with transcriptions factors and oxidized proteins. (B) The glutathione reductase system, lacking in actinomycetes, whose disulfide status is instead modulated by thioredoxin (69).

1.5 Additional agroindustrial applications of proteases and reductases

The enticing development of an enzymatic system to fully hydrolyze feather waste is not just limited to benefiting the poultry and animal feed industries (Table 1.7). The protein-rich hydrolysate may be used as a fertilizer on farms, detergent, in leather processing to de-hair products, biodegradable glue or films, and even in the degradation of prion proteins that are the causes of severe diseases such as transmissible spongiform encephalopathy (47). Enzymatic aerobic digestion of feather waste may be used as biofuel through the generation of natural gas (73).

1.6 Research objectives

Our overall goal was to explore the use of nontraditional food sources, microalgae biomass and poultry feather waste, by determining their feasibility as high protein animal feed additives. Pertaining to microalgae biomass, we sought to determine its maximal inclusion level in the diets of weanling pigs without negatively affecting swine health status and growth performance; its effect on deficiencies in the mineral profile of the pigs; and if the inclusion of additional feed additives would help recover any depressed growth performance parameters caused by algae in the diet.

With respect to poultry feather waste, we looked to improve the degradation of feather by microbial enzymes we previously identified as having been secreted during feather hydrolysis by the effective bacteria *Streptomyces fradiae* var. k11 (92, 93, 94 ,95, 96). To this end, we engineered newly identified and upregulated serine proteases and thioredoxin disulfide reductases from *S. fradiae* var. k11, characterized the enzymes, and conducted *in vitro* feather hydrolysis assays.

Table 1.7 Potential applications of microbial keratinases and/or feather hydrolytic cocktail (47).

Fields	Applications
Agroindustry	Production of feed hydrolysates
	Feed supplements
	Production of entomopathogens
	Production of nitrogen fertilizers
Pharmaceutical/Biomedical	Prion hydrolysis
	Enhanced drug delivery
	Dermatological treatments
	Cosmetics
Industry	Leather processing (tannery)
	Fiber modification (textiles)
	Detergents
	Modification of protein functionality (foods)
	Biopolymers (films, coatings, glues)
	Wastewater treatment/ waste management
Biomass/bioenergy	Bioconversion of keratinous wastes for production of biohydrogen or methane

CHAPTER TWO

Defatted and full-fat diatom microalgal biomass can partially replace corn and soybean meal in the diets of weanling pigs*

2.1 Abstract

Protein-rich microalgal biomass from biofuel production stands as a promising new animal feed source, and may serve as an alternative to corn and soybean meal (SBM) in animal diets. The objective of this study was to determine potential and limitation of a new diatom microalgal species *Staurosira sp* (or full-fat microalgae, FFA) and its defatted algal biomass (DFA) generated from biofuel production (Cellana, Kailua-Kona, HI) in replacing SBM and corn in diets for weanling pigs. In Exp. 1, 4 groups (n = 8/group) of pigs were fed a corn and SBM basal diet (BD), BD + 7.5% DFA replacing SBM, and BD + 7.5% or 15% DFA replacing corn and SBM for 6 wk. Compared with those of pigs fed the BD, ADG and G:F were lower ($P < 0.05$) in pigs fed either BD + 7.5% DFA replacing SBM only, or BD + 15% DFA ($P < 0.05$). Replacing 15% DFA resulted in lower ($P < 0.05$) fecal Cu, Se, and Zn, greater ($P < 0.01$) fecal S, Cr, Ni, Pb, Si, Sr, and Ti, and greater ($P < 0.05$) plasma Fe and Sr. In Exp. 2, 4 groups (n = 10/group) of pigs were fed the BD, BD + 10% FFA replacing corn and SBM, BD + 10% FFA + 2% fumaric acid (Univar, Morrisville, PA), and BD + 10% FFA + 50% higher levels of Cu, Se, and Zn for 6 wk. Pigs fed 10% FFA displayed lower ($P < 0.05$) ADG and G:F than those fed BD, whereas supplementing the FFA-containing diet with 2% fumaric acid, but not extra trace elements, prevented these negative effects of FFA. In conclusion, it was feasible to supplement 7.5% DFA or 10% FFA with an organic acid for a replacement of the same amount of corn and SBM in

diets for weanling pigs, without adverse effect on growth performance, biochemical status, or fecal excreta.

*Submitted to the Journal of Animal Science

2.2 Introduction

With a projected global population increase to over nine billion by 2050 (1), the availability and security of food is becoming challenging. Corn and soybean meal are two staple food sources for humans, and have also been used for biofuel production. As the primary sources of high-quality protein and energy incorporated into agricultural animal diets to meet their nutrient requirements, such usage directly competes with human consumption of these ingredients. Thus, our current allocation of substantial amount of corn and SBM as animal feed presents a serious challenge to food security and sustainable animal agriculture.

Marine microalgae have emerged as a promising alternative to corn and SBM in animal diets, owing to the rich proteinaceous feature of many species. After lipid extraction for biofuels, these microalgae generate residual biomass that shows viability to partially replace conventional protein sources in food animal diets (15). Diatoms comprise a large fraction of phytoplankton, and a new diatom microalgae species *Staurosira* is currently under investigation for biofuel production. Despite previous successful inclusions of other algae in animal diets (32, 33), potentials of this microalgal species in its full-fat form (FFA) or defatted biomass (DFA) in replacing corn and SBM in diets of weanling pigs have yet to be determined. Meanwhile, diatom microalgal biomass often contains high ash, sodium, and silicon that may affect acid base balance and body health. Supplementing the algal biomass containing diets with organic acids

such as fumaric acid, or certain trace elements may help prevent potential adverse effects of the algal biomass.

Therefore, the objectives of this study were to determine: 1) effects of 7.5 or 10% inclusion of DFA replacing various levels of corn and SBM in the diets of weanling pigs on growth performance, plasma biochemical indicators, and carcass traits; 2) responses of fecal and plasma mineral profiles to the inclusions of DFA; and 3) potential of adding fumaric acid or extra trace elements in recovering depressed growth performance due to 10% inclusion of FFA.

2.3 Materials and Methods

2.3.1 Animals and dietary treatments

Our animal experiments were approved by the Institutional Animal Care and Use Committee of Cornell University. All pigs were weanling crossbreds (Yorkshire-Landrace-Hampshire) selected from the Cornell University Swine Farm. Pigs were weaned at 4 wk of age, and allotted into treatment groups based on body weight, litter, and sex. The pigs were housed individually in pens (1 × 2.5 m) with concrete floors in a temperature-controlled barn (22 – 25°C) with a light:dark cycle of 12:12 h. In both experiments, the pigs were acclimated for 4 d on a corn-SBM basal diet (**BD1**, **BD2**). All pigs had free access to feed and water, and were monitored daily.

A preliminary experiment was conducted to determine appropriate inclusion rates of FFA and DFA in the experimental diets for replacing corn and SBM in the diets of weanling pigs. Accordingly, 32 weanling pigs (13.4 ± 1.6 kg body weight; **BW**) were selected in Exp. 1 and

divided into 4 groups (n = 8/group) and fed BD1, BD1 + 7.5% DFA replacing SBM (**7.5% DFA- A**), BD1 + 7.5% DFA replacing a combination of corn and SBM (**7.5% DFA- B**), or BD1 + 15% DFA replacing a combination of corn and SBM (**15% DFA**) for 6 wk (Tables 2.1 and A2). In Exp. 2, 40 weanling pigs (9.6 ± 0.8 kg BW) were divided into 4 groups (n = 10/group) and fed BD2, BD2 + 10% FFA replacing a combination of corn and SBM (**10% FFA**), BD2 + 10% FFA + 2% fumaric acid (Univar, Morrisville, PA) (**10% FFA + FA**), or BD2 + 10% FFA + 50% higher levels of Cu, Se, and Zn than in the premix of BD2 (**10% FFA + TM**) for 6 wk (Tables 2.1 and A3). The selection and level of Cu, Se, and Zn in the 10% FFA +TM were based on fecal and plasma mineral analyses from Exp. 1. Samples of FFA, DFA, BD1, and BD2 were submitted to commercial laboratories for proximate analysis and amino acid profiles; and all experimental diets were assayed for mineral profile (Tables 2.2 and 2.3).

2.3.2 Growth performance and sample collection

Feed refused from individual pigs were collected daily, and BW and feed intake (**FI**) were recorded biweekly to calculate ADFI, ADG, and G:F. Whole blood samples of all individual pigs were collected initially and then biweekly from the anterior vena cava using heparinized tubes (158 USP units; Vacutainer, Becton Dickinson, Franklin Lakes, NY) after an overnight fast (8 h) for assays of blood hemoglobin content and packed cell volume in Exp. 2. The collected whole blood samples were chilled on ice and centrifuged at 3000 ×g for 10 min at 4°C (GS-6KR centrifuge, Beckman Instruments, Palo Alto, CA) to prepare plasma samples for biochemical assays. In Exp. 1, fresh fecal samples from the rectum of each pig were collected once during a 12 h period, using sterile utensils at the end of study (wk 6), placed in pre-weighed

sterile 50-mL conical tubes, and stored on ice for transport to the laboratory. The wet weights of the fecal samples were determined, and the samples were stored at -80°C until freeze-drying.

2.3.3 Body lean yield predictions

In Exp. 1, each pig was subject to an ultrasound scan using an Aloka 5011 probe (Model 500V B mode scanner; Corometrics Medical Systems, Wallingford, CT) at the end of study. Animals were restrained in ventral recumbency, and the image was taken after aligning the last rib on the ultrasound grid, then guiding the probe along the midline until reaching the intercostal muscle lining. Automatic measurements of the vertebral fat and loin depths were taken, and percentage body lean yield was predicted using the AUSKey automated measuring system (Animal Ultrasound Services, AUSkey System, Ithaca, NY).

2.3.4 Fecal dry matter and mineral analyses

In Exp. 1, frozen fecal samples were placed in a lyophilizer (Freeze-dry Specialities, Osseo, MN) for 36 h, weighed to calculate the percentage of dry matter, and stored at -20°C. For mineral analyses, freeze-dried fecal samples (100 g) were thawed at room temperature and ground to pass through a 1mm screen, and plasma samples (200 μL) were thawed at 4°C. The concentrations of individual elements in both the fecal and plasma samples were measured using an inductively couple argon plasma spectrophotometer (ICAP 61E Trace Analyzer, Thermo Jarrell Ash corporation, Franklin, MA). Before analysis, samples were digested in a mixture of

HNO_3 and $HClO_4$ (9:1, volume/volume), and diluted in 5% HNO_3. Standard reference materials (No. 1573a, tomato leaves, and No. 1577b, bovine liver, National Institute of Standards and Technology, Gaithersburg, MD) were used to validate the analytical procedures (74).

2.3.5 Plasma and blood biochemical analyses

Plasma alkaline phosphatase activity was measured by the hydrolysis of *p*-nitrophenol phosphate to *p*-nitrophenol (75). The enzyme unit was defined as 1μmol of *p*-nitrophenol released per min at 30°C. Plasma alanine transaminase activity was measured using a kit as described by the manufacturer's instructions (Thermo Scientific, Waltham, MA). Plasma urea nitrogen concentration was determined by modified methods described previously (76, 77). Total triglyceride, cholesterol, and non-esterified fatty acids were analyzed using enzymatic colorimetric kits (Wako L-Type Triglyceride M, Cholesterol E, and NEFA C, respectively). Inorganic phosphorus concentration was determined using Elon (p-methyl-aminophenol sulfate) solution after de-proteination with 12.5% trichloroacetic acid (78). Packed cell volume was determined after whole-blood was drawn into heparinized microcapillary tubes (Fisher Scientific, Pittsburgh, PA), sealed, and centrifuged at 2000 × g for 12 min. Whole-blood hemoglobin concentrations were measured spectrophotometrically using the cyanomethemoglobin method, following manufacturer's instructions (Pointe Scientific).

2.3.6 Statistical analyses

Data were analyzed using the General Linear Models procedure of SAS (SAS Inst. Inc, Cary, NC). In both experiments, the main effects of dietary treatments on growth performance

and plasma biochemical measures were subjected to one-way ANOVA with time-repeated measurements (79). Duncan's multiple range test was used to compare fecal mineral levels and percent body lean yield. For all analyses, pooled SEM were listed and the significance level for differences was $P < 0.05$.

Table 2.1 Composition of basal diets 1 (Exp. 1) and 2 (Exp. 2; as-fed basis)

Item	Treatment	
	BD1[1]	BD2[2]
Ingredient, %		
Corn	65.60	67.24
Soybean meal (48% CP)	28.00	26.80
Corn oil	1.00	1.00
De-fatted diatom algae[3]	-	-
Full-fat diatom algae[3]	-	-
Plasma, spray-dried	1.50	1.50
Limestone	1.05	0.90
Dicalcium phosphate	1.50	0.99
Vitamin/mineral premix[4]	0.20	0.50
Salt	0.50	0.50
Magnesium oxide	0.05	0.05
DL-Met	-	0.01
L-Lys-HCL	0.10	-
L-Threonine	-	0.01
Antibiotic[5]	0.50	0.50
Calculated nutrient composition		
ME,[6] kcal/kg	3,274	3,151
CP, %	19.9	17.8
CF, %	4.43	4.67
Ca, %	0.84	0.89
Total P, %	0.68	0.69
Lys, %	1.20	0.96
Thr, %	0.78	0.70
Trp, %	0.24	0.19

[1]BD1 = Corn-soybean meal basal diet in Experiment 1.

[2]BD2 = Corn-soybean meal basal diet in Experiment 2.

[3]Nutrient composition of de-fatted and full-fat diatom algae were analyzed by Dairy One, INC. Ithaca, NY 14850, and Experiment Station Chemical Laboratories, University of Missouri.

[4]Vitamin and mineral premix supplied the following amounts (per kilogram of diet) : Vitamin A, 2,200 IU; vitamin D_3, 220 IU; vitamin E, 16 IU; vitamin K, 0.5 mg; biotin, 0.05 mg; choline, 0.5 g; folacin, 0.3 mg; niacin, 15 mg; pantothenic acid, 10

mg; riboflavin, 3.5 mg; thiamin, 1 mg; vitamin B_6, 1.5 mg; vitamin B_{12}, 17.5 µg; Cu, 6 mg; I, 0.14 mg; Mn, 4 mg; Zn 100 mg; Se, 0.3 mg; Mg, 0.4 mg; Fe, 80 mg.

[5]Antibiotic additive for BD1 (Tylan 10) contained tylosin (as tylosin phosphate) at 22 g/kg (Elanco). For BD2, contained 55 mg of chlortetracycline hydrochloride.

[6]Calculated based on NRC (1998).

Table 2.2 Analyzed mineral concentration of diets in Exp. 1

Item	Treatment			
	BD1[1]	7.5% DFA-A[2]	7.5% DFA-B[3]	15% DFA[4]
Macromineral, g/kg				
Ca	6.1	6.2	6.0	8.6
K	6.8	7.3	7.7	9.4
Mg	1.6	2.4	2.3	3.1
Na	2.2	3.0	3.0	6.7
P	6.2	7.4	6.8	6.8
S	1.9	2.4	2.4	3.2
Micromineral, mg/kg				
Al	132.5	197.6	193.3	205.4
As	7.9	7.8	5.4	9.9
B	227.7	243.1	227.7	245.3
Ba	0.7	1.3	0.9	1.0
Ca	0.3	0.1	0.1	0.1
Co	0.5	0.9	1.8	1.2
Cr	4.7	7.7	8.7	9.7
Cu	15.3	13.6	15.4	22.5
Fe	224.8	394.6	386.9	531.0
Mn	37.1	49.3	63.7	58.2
Mo	-	-	-	-
Ni	2.4	2.3	4.7	4.2
Pb	11.3	7.2	4.5	6.2
Se	0.1	0.1	0.1	0.1
Si	89.9	941.8	926.5	1205.9
Sr	5.7	68.4	37.0	108.0
Ti	2.7	6.3	8.2	11.5
Zn	118.1	99.4	91.3	119.9

[1] BD1 = Corn-soybean meal basal diet in Experiment 1.

[2]7.5% DFA-A = 7.5% soybean meal replaced with defatted algal biomass.

[3]7.5% DFA-B = 7.5% combination of corn and soybean meal replaced with defatted algal biomass.

[4]15% DFA = 15% combination of corn and soybean meal replaced with defatted algal biomass.

Table 2.3 Analyzed mineral concentration of diets in Exp. 2

Item	Treatment			
	BD2[1]	10% FFA[2]	10% FFA + FA[3]	10% FFA + TM[4]
Macromineral, g/kg				
Ca	6.7	8.5	5.4	6.8
K	10.4	10.2	10.4	9.9
Mg	1.8	2.1	2.3	2.0
Na	2.9	5.3	3.7	3.1
P	6.3	5.6	6.2	5.4
S	2.0	2.4	2.4	2.2
Micromineral, mg/kg				
Al	137.0	98.0	106.2	94.2
As	0.2	-	0.2	-
B	71.2	60.9	64.4	70.2
Ba	1.2	1.2	1.0	1.4
Ca	0.1	0.1	0.1	0.1
Co	0.1	0.2	0.2	0.2
Cr	1.4	1.2	1.3	1.2
Cu	12.6	10.5	12.2	11.4
Fe	212.1	268.1	280.8	240.8
Mn	27.7	25.2	28.7	28.8
Mo	0.7	0.3	0.5	0.3
Ni	1.8	1.7	1.9	1.9
Pb	0.4	1.0	0.9	0.9
Se	0.5	0.5	0.4	0.6
Si	17.7	25.0	22.0	20.6
Sr	4.1	116.3	56.3	87.2
Ti	3.0	3.5	3.8	3.2
Zn	142.6	116.3	114.0	141.8

[1]BD2 = Corn-soybean meal basal diet in Experiment 2.

[2]10% FFA = 10% corn and soybean meal replaced with full fat algae.

[3]10% FFA + FA = 10% corn and soybean meal replaced with full fat algae, and supplemented with 2% fumaric acid.

[4]10% FFA + TM = 10% corn and soybean meal replaced with full fat algae, and supplemented with 50% higher levels of trace minerals Cu, Se, and Zn than in the BD2 premix.

2.4 Results

Experiment 1

Compared with pigs fed only BD1, those fed 7.5% DFA-A or 15% DFA had lower ($P < 0.05$) overall ADG (by 11%) and G:F (by 9 and 11%, respectively; Table 2.4). Pigs fed 7.5% DFA-B had lower ($P < 0.05$) overall G:F (by 8%) than pigs fed BD1. All 4 dietary treatment groups of pigs had similar biweekly or overall ADFI, plasma alkaline phosphatase activities, plasma alanine aminotransferase activities, plasma urea nitrogen concentrations, and plasma lipid profiles of total cholesterol, total triglycerides, and total non-esterified fatty acid concentrations (Table 2.5). Additionally, no significant differences were detected in the body lean yield predictions among all 4 treatment groups (Table A5).

Inclusions of DFA elevated ($P < 0.0001$ to 0.02) fecal mineral concentrations of S, Cr, Ni, Pb, Sr, and Ti,, but decreased ($P < 0.05$) fecal Cu, Se, and Zn (Table 2.6). No treatment differences were detected for all other fecal macro- and micro-minerals (Table A4). Pigs fed 15% DFA had 22%, 54%, and 61% greater ($P = 0.03$) plasma Fe concentrations than those fed the BD1, 7.5% DFA-A, and 7.5% DFA-B, respectively. Plasma Sr concentration was elevated ($P < 0.0001$) up to 10-fold in pigs fed the DFA-containing diets, as compared to pigs fed BD1. All other plasma macro- and micro- minerals showed no significant differences across all 4 treatment groups. There was an elevation trend in the fecal dry matter concentration with the increasing inclusion of DFA in the diets, where it was 12% greater ($P = 0.08$) in pigs fed 15% DFA than those fed BD1.

Experiment 2

Compared with those fed BD2, overall ADG and G:F were 7% and 2% lower in pigs fed 10% FFA, 0.5% and 2% greater in pigs fed 10% FFA + FA, and 7% and 5% lower ($P < 0.03$) in pigs fed 10% FFA + TM, respectively (Table 2.7). There was no difference in overall ADFI, plasma inorganic phosphorus, or plasma alkaline phosphatase activity among treatment groups (Table 2.8). However, there was a marginal increase in the overall packed cell volume ($P = 0.06$) and hemoglobin concentration ($P = 0.06$) in pigs fed the FFA-containing diets. Pigs fed 10% FFA + TM had 9% greater ($P < 0.02$) at 6 wk than the pigs fed BD2, but there was also a 17% baseline difference at 0 wk.

Table 2.4 Effect of dietary defatted diatom microalgal biomass on overall growth performance of pigs in Exp. 1

Item	Treatment				SEM	Main effect, *P*-value		
	BD1[1]	7.5% DFA- A[2]	7.5% DFA- B[3]	15% DFA[4]		Diet	Week	Diet × Week
ADG[5], kg	0.88[a]	0.78[b]	0.82[ab]	0.78[b]	0.02	0.02	<0.0001	0.70
ADFI[6], kg	1.47	1.47	1.50	1.46	0.05	0.92	<0.0001	0.99
G:F[7], kg/kg	0.60[a]	0.55[b]	0.59[a]	0.53[b]	0.02	0.045	<0.0001	0.68

[a,b]Within a row, means without a common superscript differ ($P < 0.05$).

[1] BD1 = Corn-soybean meal basal diet in Experiment 1.

[2]7.5% DFA-A = 7.5% soybean meal replaced with defatted algal biomass.

[3]7.5% DFA-B = 7.5% combination of corn and soybean meal replaced with defatted algal biomass.

[4]15% DFA = 15% combination of corn and soybean meal replaced with defatted algal biomass.

[5]ADG = Average daily gain.

[6]ADFI = Average daily feed intake.

[7]G:F = Weight gain to feed ratio.

Table 2.5 Effect of dietary defatted diatom microalgal biomass on plasma biochemical measures of pigs in Exp. 1

Item	Week	Treatment				SEM
		BD1[1]	7.5% DFA-A[2]	7.5% DFA-B[3]	15% DFA[4]	
Alkaline phosphatase activity, U/L[5]	0	81.0	72.1	77.5	83.1	4.0
	6	80.9	78.0	80.4	85.8	4.1
Alanine aminotransferase activity, U/L	0	19.0	19.1	22.9	22.6	1.4
	6	18.9	17.2	19.7	20.8	1.2
Urea N, mg/dL	0	10.0	9.9	9.9	10.0	0.5
	6	15.2	14.2	14.7	15.0	0.7
Total cholesterol, mg/dL	0	64.2	56.7	56.3	58.6	5.5
	6	107.4	102.9	105.0	97.9	5.0
Total triglyceride, mg/dL	0	44.2	49.0	49.3	52.4	5.1
	6	39.4	33.6	30.8	29.3	2.9
Total non-esterified fatty acid, μmol/L	0	48.8	59.7	70.7	50.5	14.4
	6	95.9	101.1	97.7	88.9	19.8

[1] BD1 = Corn-soybean meal basal diet in Experiment 1.

[2]7.5% DFA-A = 7.5% soybean meal replaced with defatted algal biomass.

[3]7.5% DFA-B = 7.5% combination of corn and soybean meal replaced with defatted algal biomass.

[4]15% DFA = 15% combination of corn and soybean meal replaced with defatted algal biomass.

[5]The enzyme unit was defined as the amount of activity that releases 1 μmol of *p*-nitrophenol per minute at 30ºC.

Table 2.6 Effect of dietary defatted diatom microalgal biomass on fecal dry matter, as well as fecal and plasma minerals concentrations of pigs in Exp. 1

Item	Week	Treatment				SEM	Main effect, *P*-value		
		BD1[1]	7.5% DFA- A[2]	7.5% DFA- B[3]	15% DFA[4]		Diet	Week	Diet × Week
DM, %	6	28.3^{b}	31.5^{ab}	28.8^{ab}	31.9^{a}	1.2	0.08	-	-
Fecal macromineral, g/kg									
S	6	2.8^{c}	3.8^{b}	3.7^{b}	5.3^{a}	0.3	0.0002	-	-
Fecal micromineral, mg/kg									
Cr	6	12.3^{c}	19.6^{bc}	24.3^{b}	36.7^{a}	2.8	0.0003	-	-
Cu	6	103.6^{a}	82.8^{ab}	75.2^{ab}	59.2^{b}	10.0	0.03	-	-
Ni	6	9.8^{b}	11.1^{b}	12.8^{b}	19.9^{a}	1.5	0.002	-	-
Pb	6	5.8^{b}	2.8^{b}	4.1^{b}	18.8^{a}	3.1	0.02	-	-
Se	6	1.1^{a}	0.7^{b}	0.8^{b}	0.6^{b}	0.3	0.047	-	-
Si	6	0.08	0.60	1.46	1.42	0.20	0.049	-	-
Sr	6	43.4^{c}	403.5^{a}	298.5^{b}	315.3^{b}	27.6	<0.0001	-	-
Ti	6	11.5^{b}	8.7^{b}	31.0^{a}	40.3^{a}	6.3	0.008	-	-
Zn	6	669.2^{a}	547.4^{ab}	528.5^{ab}	472.3^{b}	70.4	0.04	-	-
Plasma macromineral, mg/L									
K	0	318	319	349	333	10.9	0.07	0.01	0.21
	6	300^{b}	406^{a}	401^{a}	417^{a}	6.2			
Plasma micromineral, mg/L									
Ba	0	322.6	371.7	452.0	431.0	25.7	0.26	0.94	0.13
	6	411.3^{ab}	367.0^{b}	346.7^{b}	444.1^{a}	13.8			
Fe	0	1.56	1.99	2.06	1.82	0.23	0.03	0.0002	0.13
	6	3.65^{ab}	2.61^{bc}	2.42^{c}	4.53^{a}	0.24			
Sr	0	0.03	0.04	0.04	0.04	0.01	<0.0001	<0.0001	<0.0001
	6	0.03^{c}	0.22^{b}	0.19^{b}	0.30^{a}	0.02			

[a,b,c]Within a row, means without a common superscript differ ($P < 0.05$).

[1] BD1 = Corn-soybean meal basal diet in Experiment 1.

[2]7.5% DFA-A = 7.5% soybean meal replaced with defatted algal biomass.

[3]7.5% DFA-B = 7.5% combination of corn and soybean meal replaced with defatted algal biomass.

[4]15% DFA = 15% combination of corn and soybean meal replaced with defatted algal biomass.

Table 2.7 Effect of dietary full-fat diatom microalgal biomass on overall growth performance of pigs in Exp. 2

Item	Treatment				SEM	Main effect, *P*-value		
	BD2[1]	10% FFA[2]	10% FFA + FA[3]	10% FFA + TM[4]		Diet	Week	Diet × Week
ADG[5], kg	0.75	0.70	0.75	0.70	0.03	0.03	<0.001	0.98
ADFI[6], kg	1.44	1.38	1.42	1.42	0.05	0.48	<0.001	0.94
G:F[7], kg/kg	0.52[ab]	0.51[ab]	0.53[a]	0.49[b]	0.01	0.004	<0.001	0.41

[a,b]Within a row, means without a common superscript differ ($P < 0.05$).

[1]BD2 = Corn-soybean meal basal diet in Experiment 2.

[2]10% FFA = 10% corn and soybean meal replaced with full fat algae.

[3]10% FFA + FA = 10% corn and soybean meal replaced with full fat algae, and supplemented with 2% fumaric acid.

[4]10% FFA + TM = 10% corn and soybean meal replaced with full fat algae, and supplemented with 50% higher levels of trace minerals Cu, Se, and Zn than in the BD2 premix.

[5]ADG = Average daily gain.

[6]ADFI = Average daily feed intake.

[7]G:F = Weight gain to feed ratio.

Table 2.8 Effect of dietary full-fat diatom microalgal biomass on plasma biochemical measures of pigs in Exp. 2

Item	Week	Treatment				SEM	Main effect, *P*-value		
		BD2[1]	10% FFA[2]	10% FFA + FA[3]	10% FFA + TM[4]		Diet	Week	Diet × Week
Inorganic P, mg/dL	0	10.1	9.0	8.9	9.8	0.91	0.97	0.03	0.99
	6	10.8	10.7	10.8	10.4	0.39			
Alkaline phosphatase activity, U[5]/L	0	176	170	169	169	13.3	0.94	<0.001	0.98
	6	249	264	266	264	7.9			
Packed cell volume, %	2	40.4	42.3	43.3	41.2	0.56	0.06	<0.001	0.63
	6	43.3	44.4	44.7	43.9	0.78			
Hemoglobin concentration, g/dL	2	14.2	13.6	13.9	14.2	0.57	0.06	0.01	0.48
	6	13.1	13.8	14.0	13.9	0.29			

[1]BD2 = Corn-soybean meal basal diet in Experiment 2.

[2]10% FFA = 10% corn and soybean meal replaced with full fat algae.

[3]10% FFA + FA = 10% corn and soybean meal replaced with full fat algae, and supplemented with 2% fumaric acid.

[4]10% FFA + TM = 10% corn and soybean meal replaced with full fat algae, and supplemented with 50% higher levels of trace minerals Cu, Se, and Zn than in the BD2 premix.

[5]The enzyme unit was defined as the amount of activity that releases 1 μmol of *p*-nitrophenol per minute at 30ºC.

2.5 Discussion

The main finding of Experiment 1 is that 7.5% DFA might be used to replace the same amounts of corn and SBM in diets of weanling pigs. Specifically, the replacement of a combination of corn and SBM by 7.5% DFA did not affect growth performance or health status of pigs compared to those fed BD1. However, a replacement of either SBM alone with 7.5% DFA or a combination of corn and SBM with 15% DFA decreased ADG and G:F. The amounts of SBM removed from these DFA-containing diets might exceed the tolerance of pigs. Compared to BD1, 7.5% DFA-B only reduced the SBM level by 5.1%, whereas 7.5% DFA-A and 15% DFA reduced the level by 7.5% and 9%, respectively. Because DFA contained only 19% crude protein whereas SBM contained 47.5% crude protein, the higher levels of replacements of SBM might create protein and amino acid limitations. These limitations might account for in part for the depressed growth performance. Diatoms are the major phytoplankton characterized by silica in the outer membrane of their cell walls (80, 81). Specifically, diatoms construct ornamented shells of amorphous silica that contain complex material in their frustule structure (82, 83). Studies on diatoms show that in some species, total amino acids found in the cell wall are 1.2-fold greater than those found in the cell contents. As well, certain amino acids appear to be consistently enriched in the cell wall compared to the cell contents, such as serine, threonine, and glycine (82). It will be fascinating to find out how the nutritional values of proteins in the DFA or FFA are associated with their amino acid distribution in the subcellular locations.

The interesting finding of Experiment 2 is that supplementing 2% fumaric acid into the 10% FFA diet recovered the resultant losses in ADG and G:F. Proximate analyses of the DFA

and FFA samples showed 45% and 40% ash content, respectively, and a nearly 20-fold increase in sodium, in contrast to either corn or SBM. As an alkali metal, sodium's heavy prominence in the microalgal biomass may largely skew the acid-base balance (84) of the diet and thus the pigs. As such, we supplemented the FFA-containing diet with an acid to better neutralize the electrolyte balance and decrease the stomach pH of the pigs. Previous studies have shown that the supplementation of fumaric acid to the diets of young pigs improved their ADG and feed efficiency, as well as increased the apparent ileal digestibility of several essential amino acids and minerals (85-88). Because early-weaned pigs are somewhat incapable of secreting an adequate amount of HCl to maintain a low pH in their stomachs, the addition of fumaric acid may help the buffering capacity. In this way, digestive enzymes function under more optimal environmental conditions to better digest plant protein in the diet (895, 88, 89). The positive growth performance response of pigs to the 10% FFA + 2% fumaric acid indicated an effective strategy to improve the nutrient digestion of microalgal biomass.

Pigs fed the DFA-containing diets in Exp. 1 had lower fecal Cu, Se, and Zn concentrations than those fed BD1. Because there were no such differences in plasma concentrations of these elements, the fecal reduction might implicate a relative deficiency of these elements. Plausibly, this scenario could be arisen from an overestimation of bioavailable and digestible microalgal mineral concentrations used to formulate the diets. Although the ash content of DFA was high and its mineral profile was relatively well-balanced, the bioavailability and chemical forms of the minerals in DFA remain unknown. Alternatively, these decreases might reflect either an improve absorption of the minerals by pigs fed DFA, an increase in mineral requirements by the body, or a possible elevated excretion of urinal minerals. All these changes could lead to less fecal excretion of these minerals. However, supplementing 50% more

Cu, Se, and Zn into the 10% FFA diet in Experiment 2 did not prevent the decreases of ADG or G:F. Seemingly, dietary supply or bioavailability of Cu, Se, and Zn was not a limiting factor in the algal replacement of corn and SBM. Alternatively, DFA and FFA did not share similar effect on Cu, Se, and Zn digestion or metabolism. Meanwhile, high ash contents in both DFA and FFA, along with the supplementation with extra trace minerals, in both experiments produced no signs of toxicity in the animals. The bioavailability of minerals in microalgae may be limited owing to the rigid structure of microalgal cell wall that comprises approximately 10% of the dry matter in most algae (15). Little is known about cell wall-specific mineral composition of diatom microalgae. Their minerals may be similarly presented within the cell wall, preventing effective digestion by simple-stomached animals.

Intriguingly, we observed a 22% higher of plasma Fe concentration in pigs fed 15% DFA than that of pigs fed BD1 in Exp. 1. Thus, we compared the packed cell volume and blood hemoglobin concentration of pigs fed BD2 and the FFA-containing diets in Exp. 2. Consistently, both measures were affected by diet ($P = 0.06$) and pigs fed the FFA-containing diets had numerically greater levels of these measures than those fed BD2. Likely, supplementing FFA might improve dietary iron bioavailability for hemoglobin synthesis. Because iron deficiency anemia is a practical problem for both pigs and humans and pigs are an excellent model of human nutrition, we are currently investigating the potential and mechanism in this regard. With slightly greater levels of phosphorus analyzed in the DFA-containing diets than BD1 in Exp. 1, we determined fecal total phosphorus and(or) plasma inorganic phosphorus concentrations in both experiments and found no differences among all treatment groups.

In conclusion, our results clearly illustrate that a combination of corn and SBM may be replaced by either 7.5% DFA or 10% FFA + 2% fumaric acid, without adverse effects on overall

growth performance and plasma biochemical status in weanling pigs. Our results on the fecal and plasma mineral profiles of the DFA-fed pigs are novel and may aid in understanding microalgae metabolism in animals. Future studies are needed to examine the nutrient bioavailability and chemical forms of minerals in the diatom microalgae, as well as elucidate the limiting factors in further using them as a new source of nutrients and bioactive compounds. While feed application of algae was explored as early as in the 1950's (21, 23, 24, 90), efforts in making algal biomass as a new generation of animal feed source are gaining worldwide support because of the rising global demands for alternative food and biofuel sources. A study by the United Soybean Board indicated that animal feeding in the U.S. alone used 27 million metric tons of SBM and 176 million metric tons of corn products and other ingredients in 2009-2010 (91). Although currently-available sources of microalgal biomass provide only 5,000 tons of dry matter per year (39), innovative feed applications of the defatted microalgae biomass, as shown in the present study, will actually help overcome the economical constraint of current biofuel production and generate substantial amount of mciroalgal biomass for animal feeding. Ultimately, a good portion of corn and SBM in animal diets can be spared for human consumption, which will promote sustainable animal agriculture and human food security.

CHAPTER THREE

Investigating poultry feathers- protein engineering of microbial enzymes and feather composition analysis

3.1. Summary

Feathers are a major by-product of the poultry industry. As a protein-rich substance, feathers also represent a potentially high protein animal feed additive. Incorporation of feathers into animal diets as digestible matter would help to relieve environmental pollution and sustain animal agriculture. However, their mechanical structure lends great resistance to degradation, and thus their bioavailability of nutrients is poor as a result of low digestibility. Previous research on hydrolyzing keratinous feathers suggests that microbial proteases and reductases may be key players in their effective degradation. We describe the first heterologous protein expression of three predicted serine proteases and seven predicted thioredoxin disulfide reductases from *Streptomyces fradiae* var. k11. Using casein as a substrate, we determined that all three proteases have highest activity in the alkaline range predominantly at pH11, and the higher temperature range of serine proteases, at approximately 60^{o}C. The inclusion of Ca^{2+} and Co^{2+} increased the activity of several propeptide and mature protease constructs. We roughly determined the capability of the proteases and reductases to solubilize keratin amino acids in an *in vitro* feather hydrolysis assay, with and without the inclusions of a known serine protease, Proteinase K, and a known chemical reducing agent, β-mercaptoethanol. Inclusion of both the k11 proteases and reductases with Proteinase K and β-mercaptoethanol indeed provided a synergistic enhancement of amino acid release from feather samples. Thus, it is possible that serine proteases and

reductases act cooperatively to hydrolyze feather keratin; purification of the enzymes is required for confirmation.

3.2 Introduction

Considering the continued rise in global population (1), alternative sources of food and energy are a necessary means to maintain sustainable animal agriculture and food security for humans and animals. While a co- product, feathers are surprisingly rich in protein (43), comprised of up to 90% keratin primarily as β-keratin (44). Its environmental ubiquity and proteinaceous character lends itself as an agreeable and valuable raw material to generate a nutrient-dense, eco-friendly, cheap animal feed additive (45, 46). Despite its current use in animal diets in the form of heat-treated feather meal, the bioavailability of its nutrients is poor; the use of keratin-hydrolyzing enzymes is an option to improve its digestibility.

Multiple microbial strains have shown surprising ability to solubilize feather keratin (47, 50), and *Streptomcyes fradiae* has been researched as one of the most efficient microbes to use keratin as a substrate (92, 93, 94, 95, 96). Yet, the mechanism by which these microbes use keratin as a nutrition substrate is still not fully understood. Keratinases (proteases) have been identified from certain microbes as capable of hydrolyzing keratin (46), yet purified keratinases alone lack the effectiveness of microbially expressed protein supernatants in the presence of feathers (96). The ineffectiveness of keratinases to hydrolyze feather waste individually has led many to additionally focus on the abundance of disulfide bonds in keratin. Reductases have been implicated as a necessary co-factor for initial keratin degradation, owing to its sufitolytic properties (67, 72). The release of thiol groups has been discovered during microbial fermentation, supporting the attribution that the reduction of keratin's disulfide bonds is critical to allow site-specific proteolysis to occur (58-60).

We aimed to understand if *S. fradiae* degrades keratin by a mechanism involving the cooperative action of serine proteases (E.C. 3.4.21) and thioredoxin reductases (E.C. 1.8.1.9) for the effective reduction of tightly packed disulfide and peptide bonds. Here, we describe the first heterologous protein expression of 3 predicted serine proteases and 7 predicted thioredoxin disulfide reductases from *Streptomyces fradiae* var. k11. Following their characterization, we determined their approximate capability to solubilize keratin amino acids in an *in vitro* feather hydrolysis assay, with and without the inclusions of a known serine protease, Proteinase K, and a known chemical reducing agent, β-mercaptoethanol.

3.3 Materials and Methods

3.3.1 Strains and Plasmids

Streptomyces fradiae var. k11 was obtained from collaborators in China (96). The cloning expression vectors pPICZαA and pYES2 were obtained from Invitrogen, CA). The *P. pastoris* strain X33 was acquired from Dr. Carl Batt of Cornell University.

3.3.2 Selection of the protease and reductase genes for cloning

Previous work in our lab generated a low quality 3.3 Mb draft genome of the feather-degrading bacteria, *Streptomyces fradiae* var. k11, by Illumina paired-end sequencing and annotating with the RAST server. A controlled bacterial culture induced with feathers was

conducted to isolate and identify potential enzymes involved in the keratinolytic degradation. Analysis of the supernatant with a 2D spot gel, tandem MS/MS, and quantitative 2D-LC MS/MS proteomics revealed 59 newly secreted proteins compared to the control un-induced culture, of which 16 were identified as peptidases.

From this past research, 3 of the 16 peptidases were selected for cloning based upon sequence homology to known serine proteases (E.C. 3.4.21) belonging to the S8 Proteinase superfamily (Table 3.1; Figure 3.1), as well as structural and functional similarity to Proteinase K, a commercially available serine alkaline protease. Reductase genes for cloning were selected as those classified under the "Thioredoxin reductase" subsystem (E.C. 1.8.1.9) owing to the conservation of their Cys-Ala-Thr-Cys redox active site (Figure 3.2), or conservation of signature thioredoxin domains as determined by the RAST server and ExPASy Prosite predictions.

3.3.3 Cloning of the protease and reductase genes

Secretion signaling peptide sequences were predicted by SignalP 4.0 Server (97). Protease inhibitor domains were predicted by NCBI Conserved Domain Search and Prosite (98, 99). The genomic DNA was extracted from *S. fradiae* var. k11 cells, as described by Pospiech and Neumann (100). Primers, including those for the pre-propeptide, propeptide, and mature sequences of each protease gene, were designed to contain the *Eco*RI and *Xba*I restriction enzyme sites in the forward and reverse sequences, respectively (Table 3.2). Similarly, primers were designed the whole sequences of each reductase gene, when the SignalP 4.0 did not detect the presence of secretion signaling peptide sequences.

Table 3.1 The selected *S. fradiae* var. k11 serine proteases, from the 59 newly induced proteins, and thioredoxin reductases, for cloning.

Gene Name		RAST[1] Server Identification	Length (bp)
Protease 1 (P1)	peg.64	Alkaline serine exoprotease A precursor	1,200
Protease 2 (P2)	peg.857	Alkaline serine exoprotease A precursor	1,575
Protease 3 (P3)	peg.3234	Secreted subtilisin-like protease	1,533
Reductase 1 (TrxR1)	peg.1687	Thioredoxin reductase	975
Reductase 2 (TrxR2)	peg.884	Alkyl hydroperoxide reductase protein C	555
Reductase 4 (TrxR4)	peg.885	Alkylhydroperoxidase protein D	540
Reductase 5 (TrxR5)	peg.2440	Thiol peroxidase, Bcp-type	495
Reductase 6 (TrxR6)	peg.2855	Alkyl hydroperoxide reductase subunit C-like protein	459
Reductase 7 (TrxR7)	peg.3923	Thioredoxin reductase	957
Reductase 8 (TrxR8)	peg.4078	Thioredoxin reductase	993

[1]RAST = Rapid Annotation using Subsystem Technology.

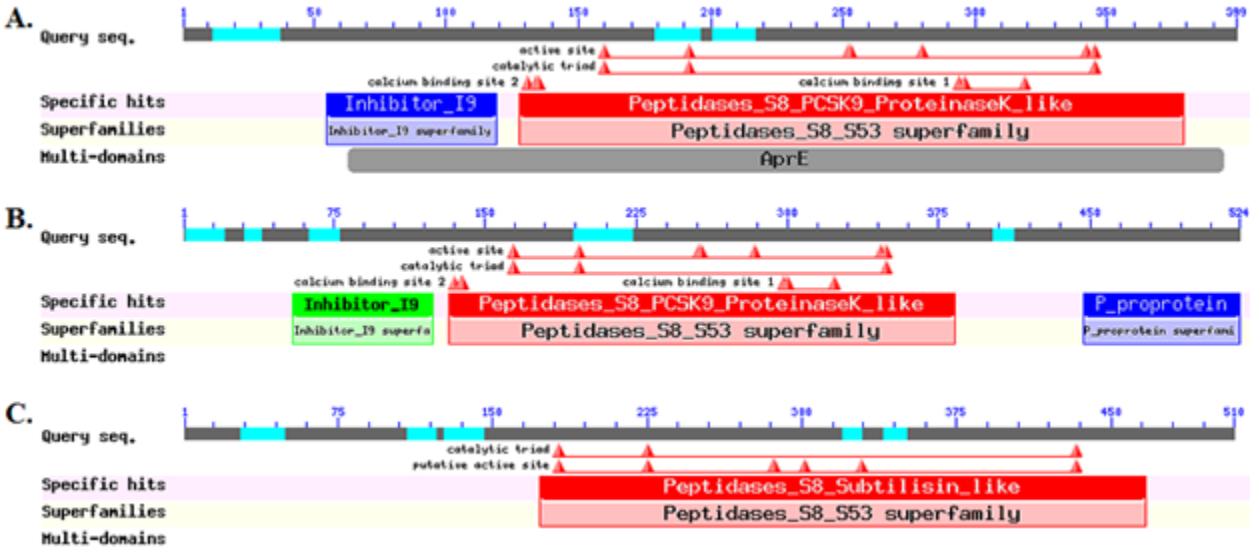


Figure 3.1 NCBI protein BLAST and ExPASy Prosite predictions for P1 (A), P2 (B), and P3 (C). Each selected protease contained a Proteinase K-like superfamily domain.

```
S.fradiae_TrxR2      TFVCPTEIAAFGKLNDEFADRDAQIL--------GVSGDSEFVHHAWRKDHDDLRDLPFP
S.fradiae_TrxR5      TPGCTKQACDFTDNLDLLAGAGYDVI--------GVSPDKPEKLAKFREK----EDLKVT
S.fradiae_TrxR6      TGVCTGELCALRDQLPRFVNEDTQLL--------AVSNDSIHTLRVFAEQ----EGLEYP
S.fradiae_TrxR4      --VSAINGCGQCLDSHEQVLRKAGVER----------DTIQEA----------------
S.fradiae_TrxR3      --PSLSQLRAFAAVAEHLHFRDAAAAIGMSQPALSGAVSALEEALGVQLLERTTRKVLLS
S.fradiae_TrxR7      --VV------HCPYCHGWEVRDEPIG------ILATGPGSLHHALLFRQL---TDDLVYF
E.coli_TrxR          --VS------ACATCDGFFYRNQKVA------VIGGGNTAVEEALYLSNI---ASEVHLI
H.sapiens_TrxR       --VS------ACATCDGFFYRNQKVA------VIGGGNTAVEEALYLSNI---ASEVHLI
S.fradiae_TrxR1      --VS------WCATCDGFFFKEHDIA------VVGGGDTAMEEATFLSRF---AKSVTVV
S.fradiae_TrxR8      --VS------SCATCDGFFFRGHDIA------VVGGGDTALEEATFLSRY---ARTVTVV
```

Figure 3.2 Multiple sequence alignment of selected reductase genes classified under the subsystem "Thioredoxin reductase" as determined by the RAST server. Comparisons to *E. coli* and human TrxRs (Accession ID YP_006158257.1 and AAB35418.1, respectively). The conserved redox active site Cys-Ala-Thr-Cys is highlighted.

Table 3.2 Oligonucleotide primer sequences, designed for the amplification of selected serine protease and reductases genes.

Primer Name	Oligonucleotide Sequence[1,2]
P1- Prepro-peptide-Forward	5’ — CCGGAATTCATGGCATCCCACAAGCGCACG — 3’
P1- Pro-peptide v1-Forward	5’ — TCCGGAATTCGCCGAGGGAAAGATCTACGGC — 3’
P1- Pro-peptide v2-Forward	5’ — ATAGAATTCGCCGCCCCGGCCGAGGGAAAG — 3’
P1- Mature-Forward	5’ — ATAGAATTCGGCGTCACCGCGTACGTCATC — 3’
P1- Reverse	5’ — GCGTCTAGAAACTCGACGACCTTGAGCAGCTTGTT CGC — 3’
P2- Prepro-peptide-Forward	5’ — CCGGAATTCATGTCAGTGATGCGTACCTCG — 3’
P2- Pro-peptide-Forward	5’ — TCCGGAATTCGCCGAGGGCGTCATCCAGTAC — 3’
P2- Mature v1-Reverse	5’ — GCGTCTAGAAAGCCGTCCCCGACGTACAGCAG
P2- Mature v2-Forward	5’ — TCCGGAATTCGGCGTCACCGCGTATGTGATC — 3’
P2- Mature v3-Forward	5’ — TCCGGAATTCGGCGTCACCGCGTATGTGATC — 3’
P2- Reverse	5’ — GCGTCTAGAAAGAACTGCAGCGCCCACGCGTC — 3’
P3- Pro-peptide-Forward	5’ — ATAGAATTCGCCCCGGCCGAGGACGTTCC — 3’
P3- Mature-Forward	5’ — TCCGGAATTCCCGCTCACCGCGGCGGAGGCC — 3’
P3- Reverse	5’ — CTAGTCTAGAAACCAGCGCACGGCGTCCAGCG — 3’
TrxR1- Forward	5’ — CCGGAATTCATGAGCGACGTCCGAAAC — 3’
TrxR1- Reverse	5’ — GCTCTAGAAAGACCGCGGGGGTCTTCTC — 3’
TrxR2- Forward	5’ — CCGGAATTCATGCTCACTGTCGGTGAC — 3’
TrxR2- Reverse	5’ — GCTCTAGAAACTCGCCGGCCAGCAGCT — 3’
TrxR4- Forward	5’ — CCGGAATTCATGTCTCTTGACGCCCTG — 3’
TrxR4- Reverse	5’ — GCTCTAGAAACTCGGAGGCGGACAGGAC — 3’
TrxR5- Forward	5’ — CCGGAATTCATGAGCGAGCGACTGCAG — 3’
TrxR5- Reverse	5’ — GCTCTAGAAATCCGCTCGCGCACGTAGC — 3’
TrxR6- Forward	5’ — CCGGAATTCATGGCGATCGAGGTCGGC — 3’
TrxR6- Reverse	5’ — GCTCTAGAAAGAGGGCGTCGAGGGCCTT — 3’
TrxR7- Forward	5’ — CCGGAATTCATGGACGTCGTGGTGGTC — 3’
TrxR7 -Reverse	5’ — GCTCTAGAAAGGCGGGGGCTTCCCCGGC — 3’
TrxR8- Forward	5’ — CCGGAATTCTGAGCGCTGACGTCCGT — 3’
TrxR8- Reverse	5’ — GCTCTAGAACGCCCCCGTGCCCGGAAG — 3’

[1]Underlined primer regions represent the *EcoRI* site in the forward primers and *XbaI* site in the reverse primers.

[2]Primers were designed to complement the multiple cloning site of expression vector pPICZαA. Identical forward primers were used to complement vector pYES2; reverse primers to complement pYES2 were identical to those for pPICZαA, with the exclusion of two adenines directly at the 3’ end of each *XbaI* site.

The genes were amplified with the PCR protocol: 94ºC for 10 min preheating, followed by 35 cycles at 94ºC for 30 s, 58-64ºC for 30 s, 68ºC for 1 min per kb of target gene, and finished with 1 cycle at 68ºC for 10 min. The annealing temperature used for each gene construct was 5ºC lower than the theoretical melting point of the primer pair, using a Bio-Rad S1000 Thermal Cycler (Philadelphia, PA). PCR products were run on 1% agarose gel and the target bands were excised and purified with Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI).

Protease gene constructs were cloned into 2 expression vectors, pPICZαA and pYES2. Reductase gene constructs were cloned into pPICZαA. Restriction endonuclease double digestions on the vectors and the successfully amplified gene inserts were performed using *Eco*RI and *Xba*I at 37ºC for 12 hours, following the manufacturer's instructional manual (Promega, Madison, WI). The pYES2 plasmid was previously constructed to contain a *Saccharomyces* α-secretion signal for similar extracellular protein expression as found in pPICZαA, using *Kpn*I and *Eco*RI upstream of the gene insertion site. The digestion products were run on a 1% agarose gel and the linearized genes were extracted, purified, and eluted in nuclease-free water using a Promega Wizard SV Gel and PCR Clean-Up Kit. T4 DNA ligase (Invitrogen, CA) was used to ligate the linearized vectors and protease genes together at 24ºC for 1 h, generating the vectors pPIC-*P1prepro*, pPIC-*P1pro1*, pPIC-*P1pro2*, pPIC-*P1mat*, pPIC-*P2prepro*, pPIC-*P2pro*, pPIC-*P2mat1*, pPIC-*P2mat2*, pPIC-*P3prepro*, pPIC-*P3pro*, pPIC-*P3mat*, and the respective pYES- vectors. The same ligation reaction was conducted on the reductase genes, generating the vectors pPIC-*TrxR1*, pPIC-*TrxR2*, pPIC-*TrxR4*, pPIC-*TrxR5*, pPIC-*TrxR6*, pPIC-*TrxR7*, and pPIC-*TrxR8*.

The recombinant plasmids were each transformed into competent *Escherichia coli* DH5α by heat-shocking with 50ng of plasmid DNA at 42ºC for 90 s, and cultured in LB at 37ºC for 1 h. Cultures were plated onto LB agar with zeocin (25μg/mL) for pPICZαA constructs and ampicillin (100μg/mL) for pYES2 constructs. Recombinant plasmids were purified from the DH5α using a QIAprep Spin Miniprep Kit (Qiagen, MD). For pPICZαA constructs, sequence-verified recombinant plasmids were linearized with the *Pme*I restriction endonuclease (Invitrogen, CA), according to the manufacturer's instructional manual. To transform into *P. pastoris* X-33, competent cell preparation and the electroporation procedure were followed according to the Electro Cell Manipulator 600 Electroporation Protocol 505 for cell line *P. pastoris* (BTX Division of Genetronics). Electroporation contents were spread on YPD plates with 100μg/ml zeocin and incubated for 3 days at 30ºC. To transform into *S. cerevisiae* INVSc1, competent cell preparation and the heat-shock procedure were followed according to the Invitrogen pYES2 manual. Heat-shock contents were spread on uracil-deficient selective media plates (Sc-U) with 2% glucose and incubated for 3 days at 30ºC.

3.3.4 Expression of recombinant enzymes in P. pastoris X33 and S. cerevisiae INVSc1

Mut^+ clones grown on the YPD with zeocin, and Ura^- clones grown on the Sc-U media, were confirmed by PCR using gene-specific primers. PCR-confirmed clones in *P. pastoris* and *S. cerevisiae* were inoculated into 15mL BMGY and 15mL Sc-U with glucose media, respectively, and cultured for 2 days at 30ºC with constant shaking at 250rpm. In pPICZαA plasmids cloned into *P.* pastoris, the *AOX1* promoter allows for strong methanol-inducible expression in yeast (101, 102, 103).

To induce protein expression in *P. pastoris*, cultures were centrifuged at 1,500 ×g for 10 minutes, and the cells were transferred to 50mL BMMY at an OD_{600nm} of 1.0. For continual induction, methanol was added every 24h to a final concentration of 0.5% for a total of 4 days. Samples of 5mL were collected at 0, 24, 48, 72, 96h. In pYES2 plasmids cloned into *S. cerevisiae*, the *GAL1* promoter in pYES2 allows for inducible expression in yeast, whereby protein expression is induced by the inclusion of galactose and repressed by glucose (104, 105).

To induce protein expression in *S. cerevisiae*, cultures were centrifuged at 1,500 ×g for 10 minutes, and the cells were transferred to 50mL Sc-U with 2% galactose at an OD_{600nm} of 0.4. Samples of 5mL were collected at 0, 24, 48, 73, and 98h.

To analyze evidence of protein expression, culture supernatants were run through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% stacking and separating gels. Proteins were stained with 0.2% Coomassie brilliant blue R-250 (103). Protein was quantified spectrophotometrically following the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL).

3.3.5 Assay of proteolytic activity, and enzyme characterization

To measure protease activity, azo-casein hydrolysis assays were conducted as previously described[16,17]. A time-course assay was initially conducted with 100µg/ml enzyme supernatant from extracellular expression in either *P. pastoris* or *S. cerevisiae*, and was added to a final 50mM glycine-NaOH, pH 9.0. A negative control was used, consisting of *P. pastoris* with vector pPICZaA and *S. cerevisiae* with vector pYES2, respectively. An additional control included 1µg/ml purified and lyophilized Proteinase K from *Engyodontium album* (Sigma, USA).

All samples were conducted in duplicates, and analyzed in duplicates. Samples were pre-warmed and final 1% azo-casein substrate was added before incubation at 50^{o}C for 30min. Ten percent TCA was used to stop the reaction, and samples were centrifuged for 10 min at 13,000 ×g. Inactive blanks of each sample were conducted by adding the TCA before the substrate. Samples were read in a 96-well clear bottom plate at A440nm, with pathlength correction. One Unit is defined as an increase in the absorbance at 440nm by 0.01 per 30 min. From these results, protease expression in *P. pastoris* showed extremely high background levels, indistinguishable from control samples of the empty vector, pPICZαA. As such, additional enzymatic characterizations were conducted on *S. cerevisiae* samples.

Influence of pH on azo-casein protease activity was determined over the pH range 7-11, using the following buffers at a final concentration of 50mM: KH_2PO_4-NaOH (pH 7), Tris-HCl (pH 8), glycine-NaOH (pH 9-11). Optimal expression time point was used for each protease construct. To determine the temperature profile, protease activity was tested at the optimal pH of each protease construct, from 37 to 70^{o}C. Inclusion of several divalent metal cations and chemical reagents were tested on proteolytic ability, using azo-casein as a substrate. The effect of 5mM Ca^{2+} ($CaCl_2$), Co^{2+} ($CoCl_2$), Cu^{2+} ($CuSO_4$), or Zn^{2+} ($ZnCl_2$), or 1mM ethylenediaminetetracetic acid (EDTA) or phenylmethanesulfonyl fluoride (PMSF) were tested by incubating the enzyme supernatants in 50mM glycine-NaOH buffer containing the added compounds, with 1% azo-casein.

Proteolytic ability of the recombinant proteases to hydrolyze a synthetic peptide, *N*-succinyl-Ala-Ala-Pro-Phe-pNA (AAPF, Sigma, USA), was studied under optimal pH and temperature conditions. A stock solution of AAPF was prepared in dimethyl sulfoxide (DMSO). AAPF (1mM) was assayed at 60^{o}C, pH 11 for 30min. The change in absorbance at 410 nm was

measured as the liberation of *p*-nitroaniline. One Unit was defined as the amount of enzyme that liberated 1uM of *p*-nitroaniline per minute. All samples were conducted in duplicates, and analyzed in duplicates.

3.3.6 Assay of reductase activity, and enzyme characterization

Activity of the recombinant TrxR enzymes was determined using 5,5'-dithiobis(2-nitrbenzoic acid) (DTNB), whereby the colorimetric production of 2-nitro-thiobenzoic acid (TNB) by GSH is measured spectrophotometrically at 412nm (106). Reductase converts GSSG into GSH in the presence of NADPH; GSH subsequently reduces DTNB to TNB

$$\text{NADPH} + \text{H}^+ + \text{GSSG} \xrightarrow{\text{reductase}} \text{NADPH}^+ + 2\text{GSH}$$

$$\text{GSH} + \text{DTNB} \rightarrow \text{GSTNB} + \text{TNB}.$$

All samples were conducted in duplicates, and analyzed in duplicates. In a total volume of 200 µl, reaction mixtures contained 100mg/ml recombinant enzyme supernatant, 0.1M potassium phosphate buffer, pH 7.6, 0.5mM EDTA, 0.75mM DTNB, 0.1mM NADPH, and 1mM GSSG. The addition of GSSG noted the initiation of the reaction, and was read before and after 2 minutes of GSSG addition. One unit of activity was arbitrarily defined as the change in absorbance by 0.1 per minute at 412nm. To determine the approximate optimal temperature, samples were run at pH 7.6, at 24^{o}C, 37^{o}C, and 55^{o}C. To determine the optimal pH, samples were run at their respective optimal temperatures, at pH 4.5, 5.0, 6.0, 7.6, and 8.0.

3.3.7 In vitro feather hydrolysis assay

In vitro hydrolysis assays were conducted on 10mg down feather samples to determine if the recombinant serine proteases and reductases from the feather-degrading microbe *S. fradiae* var. k11 were capable of effective hydrolysis both visually and biochemically, as defined by the presence of soluble amino acids. The assay was conducted using two separate incubation steps. The first incubation step incorporated different combinations of the recombinant enzymes with a feather. Feather samples were washed in incubation buffer, to remove traces of the recombinant enzymes and chemical compounds in the buffer, before proceeding to the second step. The second step included the washed feather samples and 100µM of the commercially-available serine protease, Proteinase K. In order to determine differential effects from the recombinant enzymes in step 1, the components of step 2 were identical for all feather samples.

Incubations were carried out in 1.7ml microcentrifuge tubes; ten milligrams of single down feathers were placed in each tube. In Step 1, experimental samples of 100mM enzyme supernatant were added to the microcentrifuge tubes with buffer containing 30mM Tris-HCl, 20mM EDTA, 10mM NaCl, pH 7.6, for a total volume of 1ml. Experimental samples included the individual addition of a k11 protease mix, k11 reductase mix, or a combination of k11 proteases and reductases. Control samples included buffer only and supernatant from the yeast hosts containing empty vectors, WT X33/pPICZαA and WT INVSc1/pYES2. In addition to these five samples, a second set of the same five samples were also tested with the inclusion of 50mM β-mercaptoehanol, a strong chemical reducing agent with known ability to hydrolyze difulside bonds. Samples were incubated at 37ºC × 250 rpm x 4h. After Step 1, supernatant samples were collected for amino acid release analysis, and microcentrifuge contents were

visualized in petri dishes. Feather samples were washed a minimum of 3 times in the same Tris-HCl buffer, to ensure that all enzyme and chemical remnants from Step 1 were removed. Feather samples were placed into new mcirocentrifuge tubes with the same Tris-HCl buffer, and 100µM Proteinase K. Again, samples were incubated at 37^{o}C × 250 rpm x 4h, and microcentrifuge contents were visualized in petri dishes.

The presence of soluble, free amino acids from incubation supernatant samples collected after Step 1 were analyzed, to help determine if feather hydrolysis could be detected biochemically, via derivitization with o-pthaldialdehyde (OPA). An equal amount of supernatant and OPA reagent (1.0 mg/ml OPA in borate buffer, pH 10.5, and 0.4% β-mercaptoethanol as the sulfhydryl moiety) were combined and read at 340nm. A serial dilution of known concentrations of amino acid standard (Sigma, USA) were used to determine the concentration of amino acids.

3.4 Results

3.4.1 Cloning into yeast

The three selected protease genes (P1, P2, and P3), along with their respective propeptide and mature constructs, were successfully cloned and expressed in both *P. pastoris* and *S. cerevisiae* (Figures 3.3a and 3.4a). The SDS-PAGE showed low expression of the proteins. Seven out of 8 TrxR genes were successfully cloned and expressed in *P.* pastoris (Figures 3.3b and 3.4b). PCR confirmations of the gene constructs homologously recombined into *P. pastoris* and episomal in *S. cerevisiae* showed successful incorporation of the plasmid into the respective yeast strains.

3.4.2 Protease activity

Initially, the successfully amplified protease genes were cloned into *P. pastoris* X33 and tested for their ability to hydrolyze azo-impregnated casein during a time course assay. The control vector pPICZαA in *P. pastoris* had either similar, or higher caseinolytic activity than all recombinant proteases at any given expression time point (Figure 3.5). As such, all gene constructs were subsequently cloned into an alternative host expression system, *S. cerevisiae*, with the plasmid pYES2 altered to include the *S. cerevisiae* α-signaling peptide sequence for extracellular protein expression.

A time course assay showed that protein expression at 98 h had the highest consistent protease activity for most protease constructs among all other time points, as compared to the negative control INVSc1/pYES2 (Figure 3.6). P1 propeptide v1 and P2 mature v2 were exceptions, and showed highest protease activity at 73 h of expression. The optimal pH of the recombinant proteases was pH 11.0, with the exception of INVSc1/pYES2 (pH 8.0) and P1 propeptide v1 (pH 9.0; Figure 3.7). The optimal temperature, tested from 37ºC to 70ºC, was 60ºC for all recombinant proteases, except for INVSc1/pYES2 and P1 propeptide v1, which both showed highest proteolytic activity at 70ºC (Figure 3.8).

The effect of different metal ions and chemical inhibitors showed that Zn^{2+} and PMSF inhibited relative activity of each protease construct, including Proteinase K (Table 3.3). The inclusion of Zn^{2+} inhibited activity from 34% for P2 Mature v1, up to 65% of activity for P1 Mature as well as the negative control WT INVSc1/pYES2. PMSF at 1mM inhibited nearly 100% of activity for every protease, with the highest residual activity remaining at 15% for Proteinase K. EDTA either had minimal to no effect on relative activity (P1 Mature and P2 Pre-

propeptide), or up to an increase in activity by 43% for P1 Propeptide v1 and P3 Propeptide. However, activity was also increased by 37% for the negative control WT INVSc1/pYES2. A similar effect was shown by the inclusion of 5mM Cu^{2+}, whereby it decreased activity by no more than 24% (P3 Pre-propeptide) and increased activity by 67% for both the negative control WT INVSc1/pYES2 and P1 Propeptide v1. The addition of 5mM Co^{2+} also increased activity for each protease construct, by up to 257% (P1 Propeptide v1), as well as 147% for WT INVSc1/pYES2. Calcium decreased relative activity for all constructs of P3, and significantly increased the activities of P1 Propeptide v1 (by 98%) and P2 Mature v1 (132%).

No recombinant protease was capable of any hydrolysis of the synthetic peptide AAPF (data not shown).

3.4.3 Reductase activity

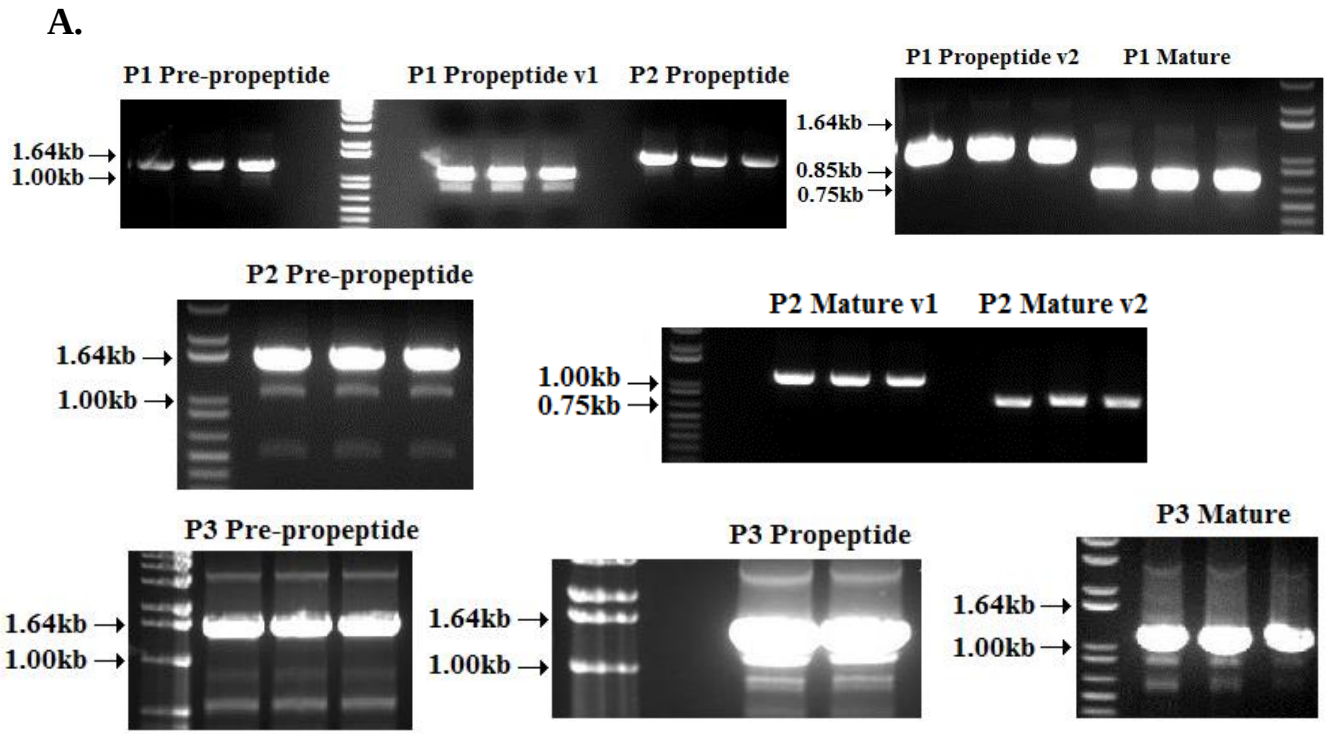
At pH 7.6, samples of 100mg/ml TrxR6, TrxR7, and TrxR8 showed appreciable reductase activity (17.5, 21, 22 mU/ml, respectively) in comparison to 1mM glutathione reductase at 28 mU/ml (Figure 3.9). Samples of TrxR1, 2, 4, and 5 showed no higher reductase activity than the negative control, WT X33/pPICZαA. The optimal pH between 4.5 and 8.0 was pH 7.6 for all TrxR constructs except TrxR1, 2, and 5, whose pH optima was 8.0 (Figure 3.10). The optimal temperature among 24ºC, 37ºC, and 55ºC was 37ºC for TrxR2, 4, and 7, and 24ºC for TrxR1, 5, 6, and 8 (Figure 3.11). At 55ºC, relative activity was significantly lowered by 25% up to 72%.

3.4.4 In vitro feather hydrolysis

After Step 1 of incubation with the various *S. fradiae* enzymes heterologously expressed in yeast, partial visual degradation was apparent from feather samples treated with β-mercaptoethanol alone, β-mercaptoethanol + k11 proteases, and β-mercaptoethanol + k11 reductases and proteases (Figure 3.12). Qualitatively, it appeared that the feather sample treated with β-mercaptoethanol + k11 reductases and proteases had the most visual separation of feather vanes from the main shaft. After Step 2 incubation with 100μM Proteinase K, all feather samples showed varying degrees of degradation. The feather treated with β-mercaptoethanol in Step 1 appeared to have the least remnants of feather remaining, as evidenced by the lack of downy vanes on the feather.

After Step 1 incubation, the soluble amino acid concentration of supernatant samples was determined via o-pthaldialdehyde derivitization (Figure 3.13). Feather samples treated with buffer only, WT yeast, k11 reductases, k11 proteases, k11 reductases and proteases, β-mercaptoethanol, or β-mercaptoethanol + k11 reductases all showed less than 20μmoles/ml release of amino acids. β-mercaptoethanol + WT yeast showed 29μmoles/ml, while β-mercaptoethanol + k11 proteases showed 100μmoles/ml. The complete combination of β-mercaptoethanol + k11 reductases and proteases showed the most release of amino acids at 113μmoles/ml.

Figure 3.3 PCR amplification of (A) serine protease and (B) TrxR genes, from isolated *Streptomyces* fradiae var. k11 genomic DNA, using designed oligonucleotide primers. P1 Pre-propeptide had an expected length of 1,200bp; P1 Propeptide v1, 1,095bp; P1 Propeptide v2, 1,086bp; P1 Mature, 741bp; P2 Pre-propeptide, 1,575bp; P2 Pro-peptide, 1,460bp, P2 Mature v1, 1,107bp; P2 Mature v2, 747bp; P3 Pre-propeptide, 1,533bp; P3 Pro-peptide, 1,431bp; P3 Mature, 1,143bp. TrxR1 had an expected length of 991bp; TrxR2, 571bp; TrxR4, 556bp; TrxR5, 511bp; TrxR6, 475bp; TrxR7, 973bp; TrxR8, 1,009bp.


A.
P1 Pre-propeptide
P1 Propeptide v1
P2 Propeptide
1.64kb
1.00kb
P1 Propeptide v2
P1 Mature
1.64kb
0.85kb
0.75kb
P2 Pre-propeptide
1.64kb
1.00kb
P2 Mature v1
P2 Mature v2
1.00kb
0.75kb
P3 Pre-propeptide
1.64kb
1.00kb
P3 Propeptide
1.64kb
1.00kb
P3 Mature
1.64kb
1.00kb

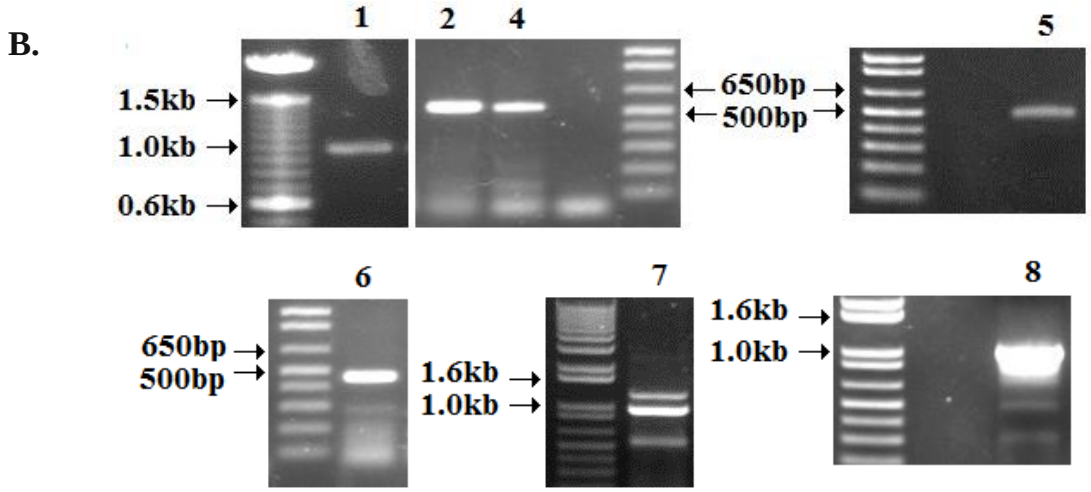
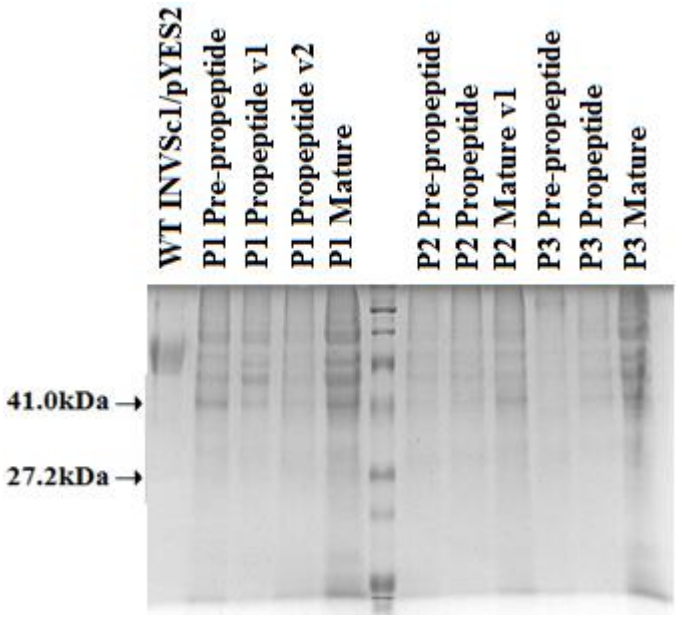
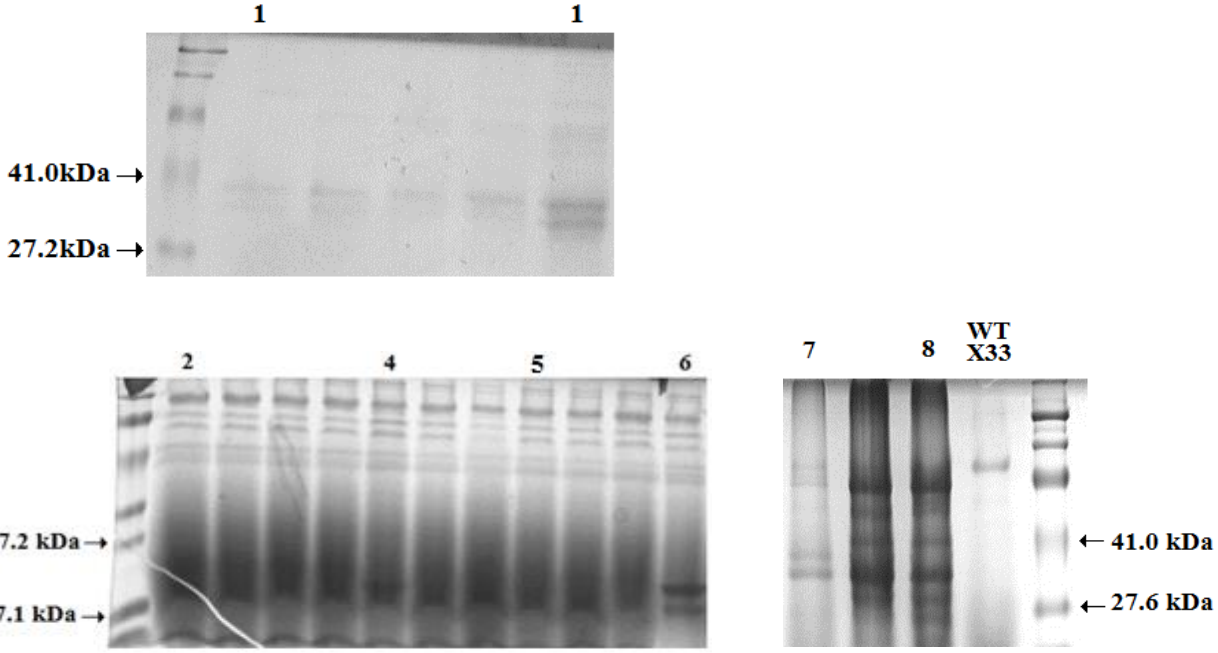

B.
1
2
4
1.5kb
1.0kb
0.6kb
650bp
500bp
5
6
650bp
500bp
7
1.6kb
1.0kb
8
1.6kb
1.0kb

Figure 3.4 SDS-PAGE gels from each protease and TrxR transformants, Protease samples were expressed *S. cerevisiae* INVSc1 supernatants after 96h of expression. TrxRs samples were expressed *P. pastoris* X33 supernatants after 96h of expression. A volume of 250µl supernatant was precipitated using tricholoroacetic acid for each transformant. P1 Pre-propeptide had an expected size of 44.4kDa; P1 Propeptide v1, 40.5kDa; P1 Propeptide v2, 40.2kDa; P1 Mature, 27.4kDa; P2 Pre-propeptide, 58.3kDa; P2 Pro-peptide, 54.0kDa, P2 Mature v1, 41.0; P2 Mature v2, 27.6; P3 Pre-propeptide, 56.7kDa; P3 Pro-peptide, 52.9kDa; P3 Mature, 42.3kDa. TrxR1 had a size of 36.5kDa; TrxR2, 20.9kDa; TrxR4, 20.4kDa; TrxR5, 18.7kDa; TrxR6, 17.4kDa; TrxR7, 35.8kDa; TrxR8, 37.1kDa.

A.


WT INVSc1/pYES2
P1 Pre-propeptide
P1 Propeptide v1
P1 Propeptide v2
P1 Mature
P2 Pre-propeptide
P2 Propeptide
P2 Mature v1
P3 Pre-propeptide
P3 Propeptide
P3 Mature
41.0kDa
27.2kDa

B.


1
1
41.0kDa
27.2kDa
2
4
5
6
27.2 kDa
17.1 kDa
7
8
WT X33
41.0 kDa
27.6 kDa

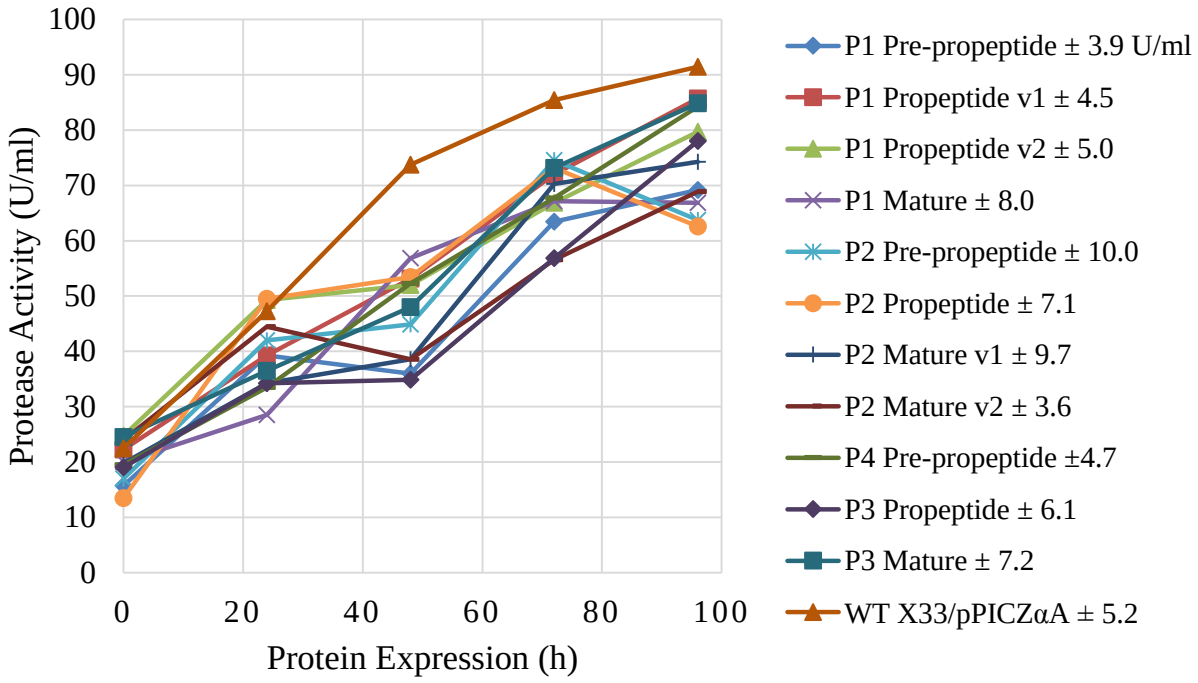


Figure 3.5 Time course assay of recombinant protease expression, up to 98h in *P. pastoris* X33. Protease activity was determined by the hydrolysis of azo-casein. Average standard deviations are shown in the legend.

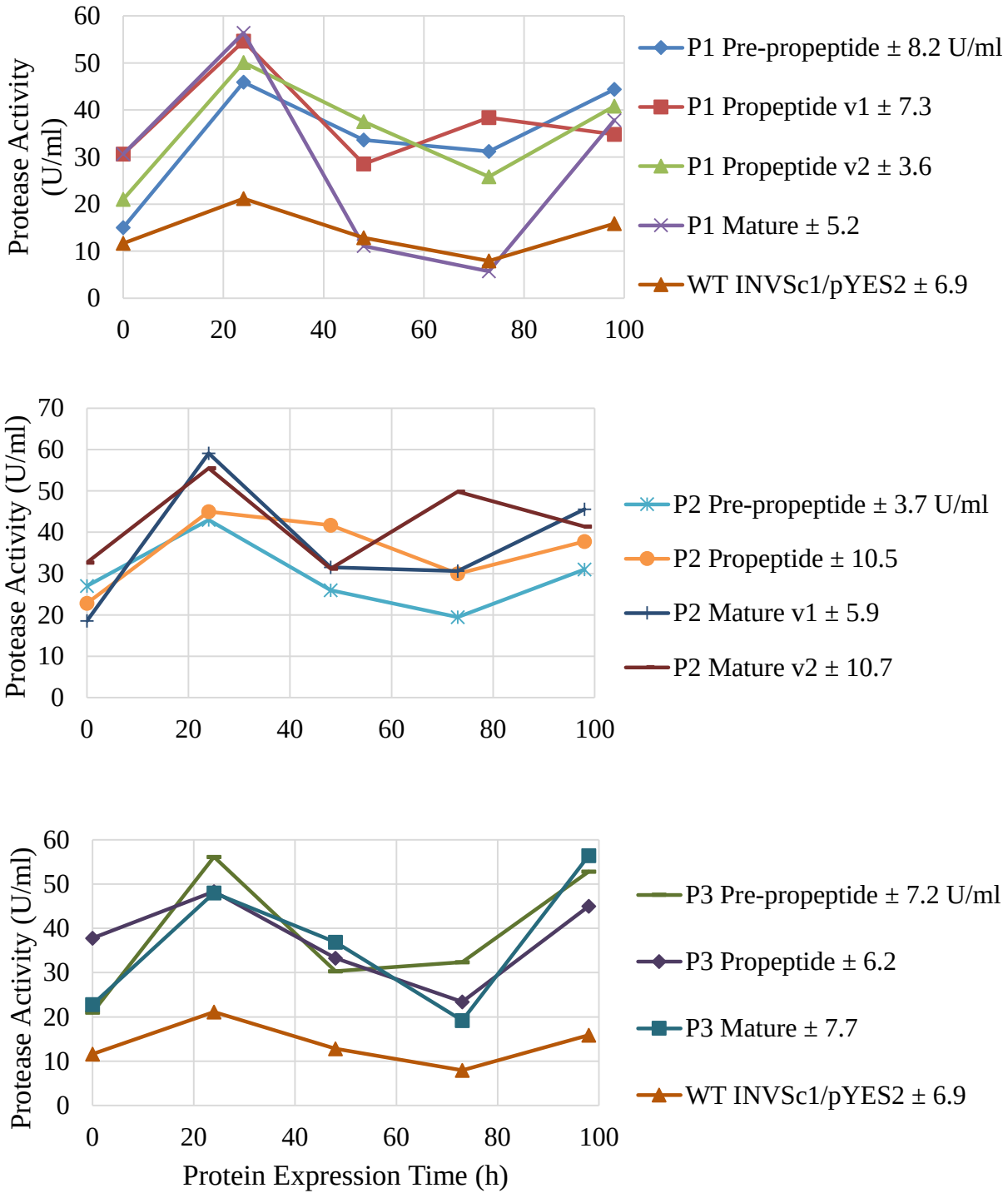


Figure 3.6 Time course assay of recombinant protease expression, up to 98h in *S. cerevisiae* INVSc1. Protease activity was determined by the hydrolysis of azo-casein. Average standard deviations are shown in the legend.

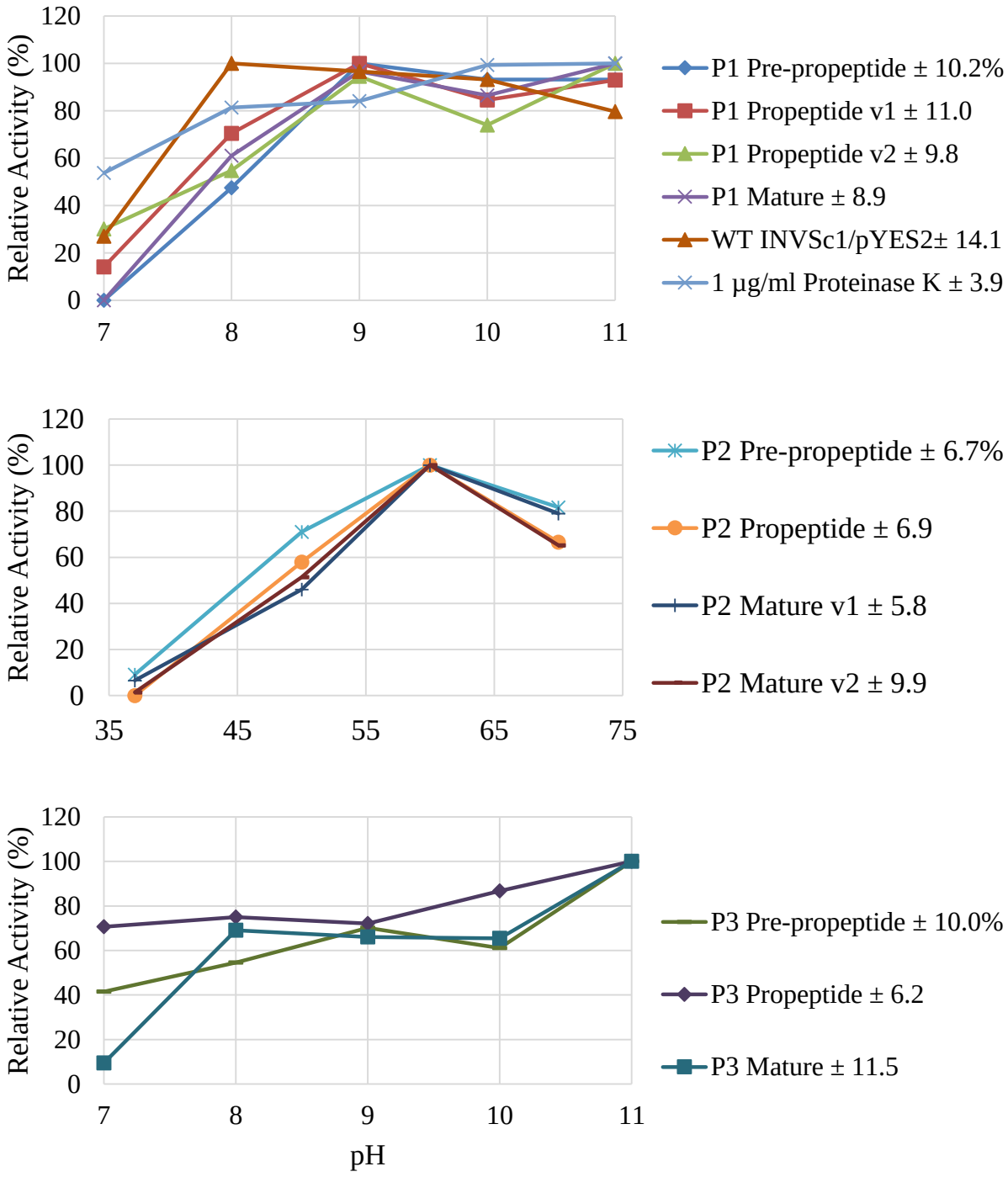


Figure 3.7 pH profile of recombinant protease expression in *S. cerevisiae* INVSc1, expressed for 98h. Protease activity was determined by the hydrolysis of azo-casein. Average standard deviations are shown in the legend.

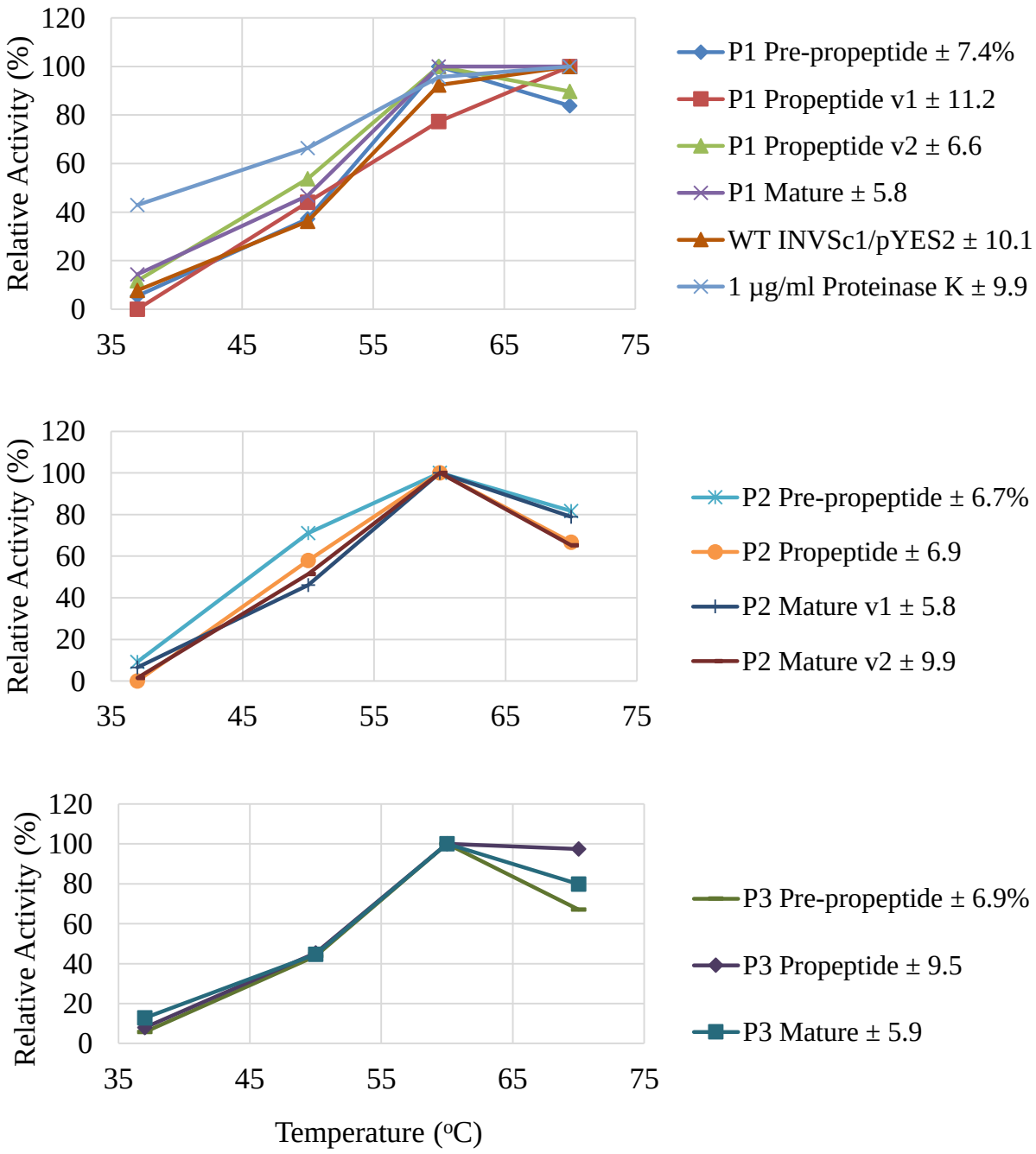


Figure 3.8 Temperature profile of recombinant protease expression in *S. cerevisiae* INVSc1, expressed for 98h. Protease activity was determined by the hydrolysis of azo-casein. Average standard deviations are shown in the legend.

Table 3.3 Relative activity of recombinant proteases, WT INVSc1/pYES2, and 1µg/ml Proteinase K with the inclusion of various metal ions and chemical inhibitors. Standard deviations are shown in the legend.

	Treatment						
Enzyme	None	Ca^{2+}	Co^{2+}	Cu^{2+}	Zn^{2+}	EDTA	PMSF
P1 Pre-propeptide	100	96 ± 5	286 ± 15	97 ± 4	49 ± 3	111 ± 5	0 ± 0
P1 Propeptide v1	100	198 ± 11	357 ± 24	167 ± 4	60 ± 5	143 ± 2	0 ± 0
P1 Propeptide v2	100	101 ± 4	343 ± 31	122 ± 6	59 ± 4	135 ± 6	0 ± 0
P1 Mature	100	108 ± 3	311 ± 25	137 ± 5	35 ± 4	89 ± 7	0 ± 1
P2 Pre-propeptide	100	101 ± 1	194 ± 17	128 ± 6	49 ± 8	98 ± 10	0 ± 0
P2 Propeptide	100	116 ± 4	349 ± 29	100 ± 9	60 ± 4	132 ± 14	0 ± 0
P2 Mature v1	100	232 ± 16	259 ± 28	93 ± 9	66 ± 1	111 ± 6	0 ± 1
P2 Mature v2	100	119 ± 6	299 ± 22	83 ± 7	49 ± 5	114 ± 5	0 ± 1
P3 Pre-propeptide	100	81 ± 8	218 ± 16	76 ± 2	38 ± 8	103 ± 8	0 ± 0
P3 Propeptide	100	85 ± 6	237 ± 20	120 ± 3	41 ± 5	143 ± 11	8 ± 0
P3 Mature	100	95 ± 3	210 ± 18	98 ± 10	41 ± 9	101 ± 8	3 ± 1
WT INVSc1/pYES2	100	103 ± 5	247 ± 19	167 ± 9	35 ± 3	137 ± 5	0 ± 0
1 µg/ml Proteinase K	100	120 ± 2	164 ± 3	153 ± 3	54 ± 5	124 ± 4	15 ± 4

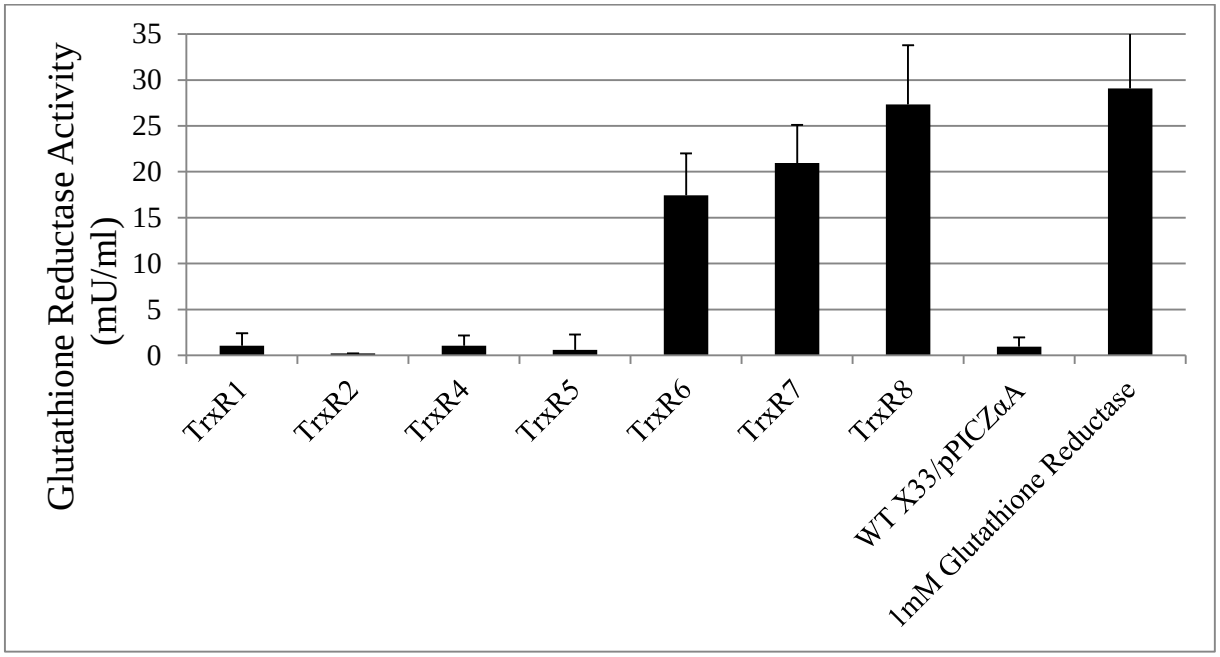


Figure 3.9 Reductase activity of transformants from each TrxR gene at pH 7.6.

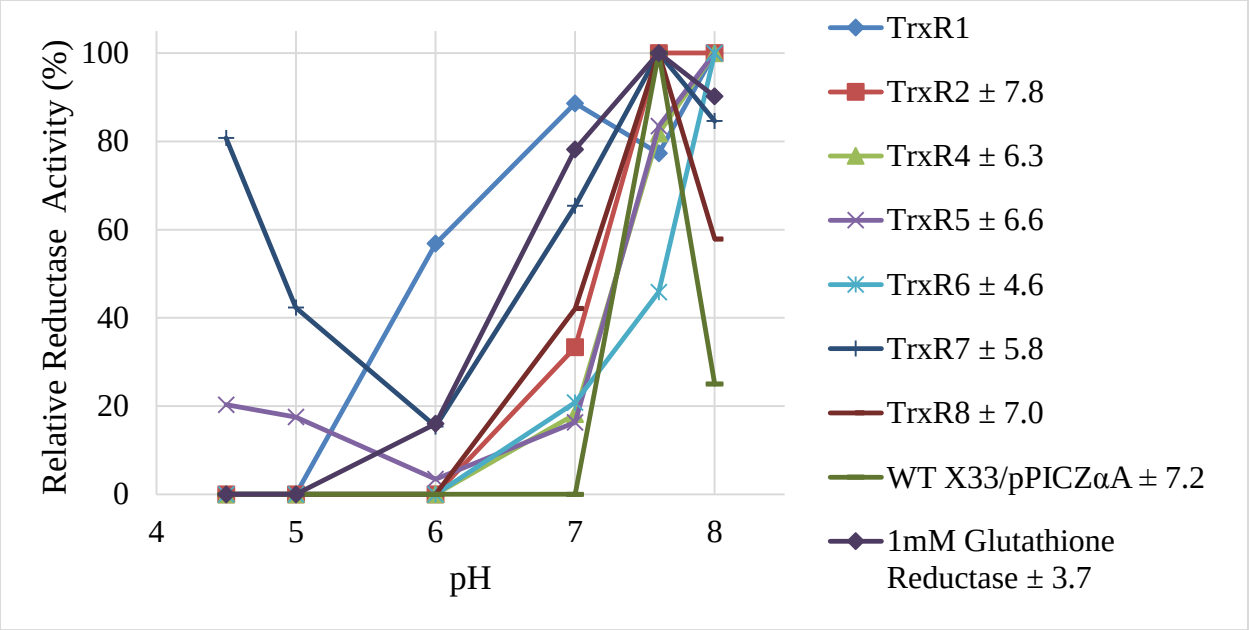


Figure 3.10 Relative reductase activity (%) pH profile of recombinant TrxRs. Average standard deviations are shown in the legend.

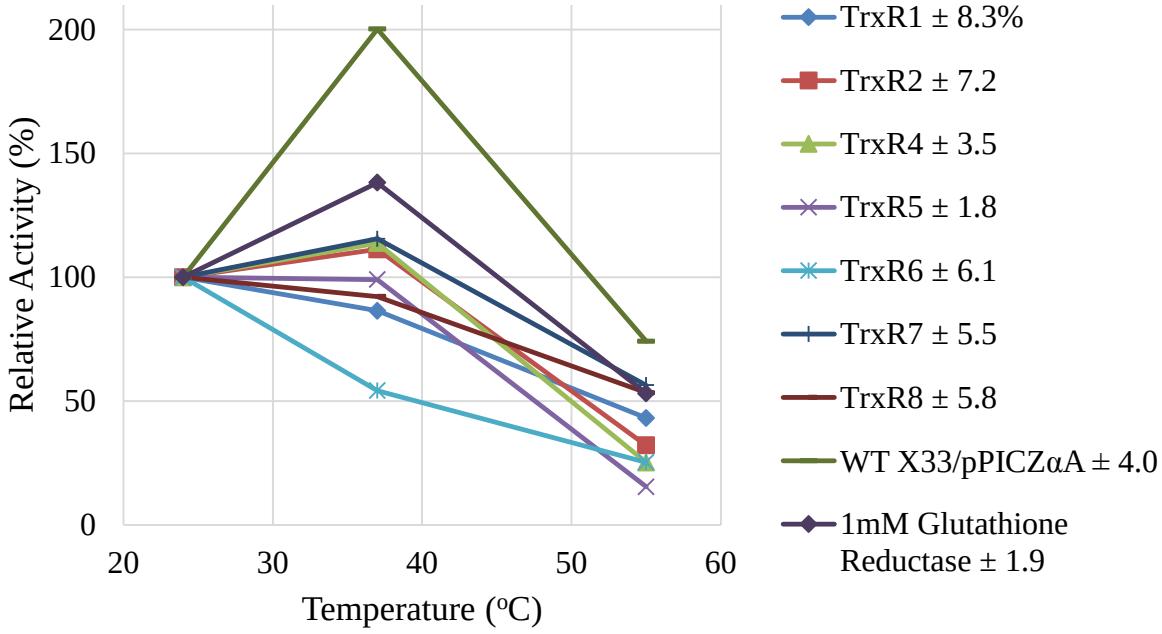


Figure 3.11 Relative reductase activity (%) temperature profile of recombinant TrxRs. Average standard deviations are shown in the legend.

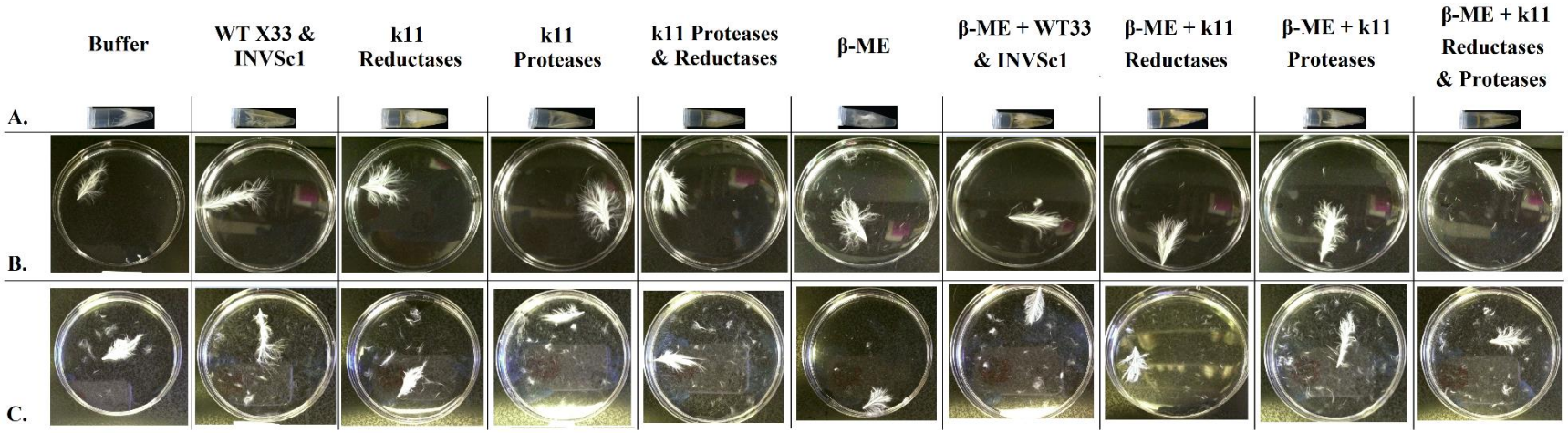


Figure 3.12 Visual hydrolysis of *in vitro* feather assay (A) before incubation in Step 1, in microcentrifuge tubes, (B) after Step 1 incubation with the labelled enzymes, and (C) after Step 2 incubation with 100µM Proteinase K.

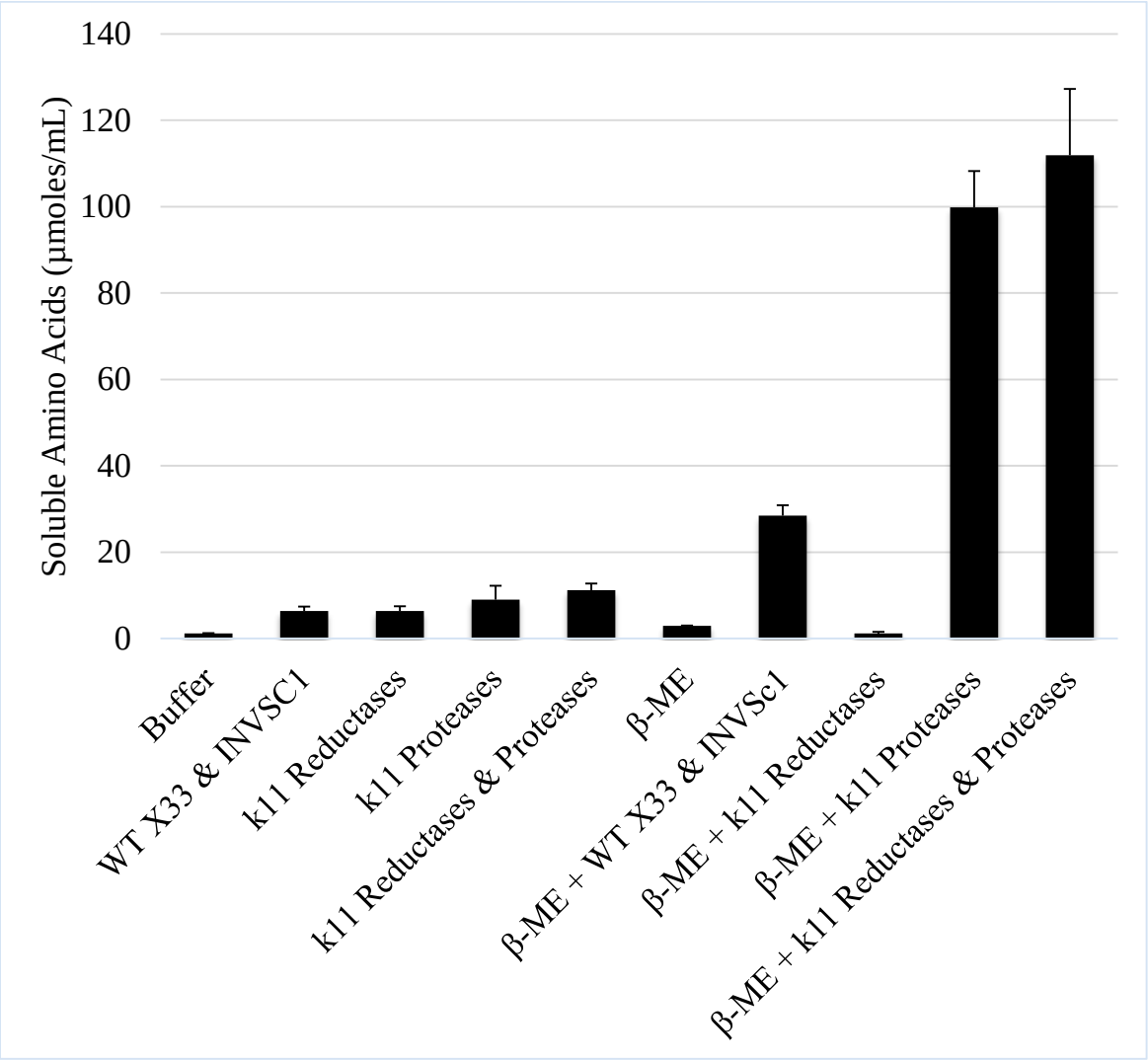


Figure 3.13 Soluble amino acids after Step 1 incubation with the labelled enzymes. Free amino acids were detected by the presence of primary amines during derivatization with o-pthaldialdehyde.

3.5 Discussion

All protease and reductase constructs were successfully amplified by PCR from the *S. fradiae* var. k11 genome and cloned into yeast, except for TrxR3. Sequence analysis showed that all 3 proteases contained the S8 peptidase superfamily domain, the same domain found in a commercially-available keratinolytic serine protease, Proteinase K. Proteinase K is isolated from the fungus *Engyodontium album*, most known for its digestion of native α-keratin (111, 112, 113). The activity of P1, P2 and P3 enzyme constructs were all lower than that of the positive control 1µg/ml Proteinase K, but showed highly similar optimal pH and temperature profiles. During experimentation in the *in vitro* feather hydrolysis assay, Step 2 included the sole incubation of the pre-treated feathers with Proteinase K, and all feather samples showed visual feather breakdown after 4 hours. A mixture of P1, P2, and P3 released the high levels of soluble amino acids with the inclusion of β-mercaptoethanol and k11 TrxRs, in comparison to any other combination of k11 proteases alone, k11 reductases alone, or β-mercaptoethanol alone. This possibly indicates that: P1, P2, and P3 require purification for enhanced caseinolysis and additional keratinolysis for more precise comparison to the purified Proteinase K; they have the potential to be the predominant keratinases showing upregulation in *S. fradiae* var. k11 culture with feathers. Further experimentation is required, involving purification of the proteases and reductases, to confirm the characterizations and determine if the synergistic effect of k11 serine proteases with reductases is indeed capable of keratinolysis. Nevertheless, the use of unpurified proteases and reductases shows promise in being an effective system for keratinolysis in feathers.

The sequence for TrxR3 was incomplete and annotated at the 3’ end, and thus it was not possible to determine the 3’ DNA sequence for primer designs. An additional primer further

downstream of the 3' end of the gene was designed in order to attempt PCR amplification and subsequent sequencing. However, PCR amplification was unsuccessful; optimized primers for this gene had melting temperatures of over 73^{o}C, which may have contributed to the difficulty in PCR amplification from the genomic DNA.

After transformation of the protease constructs into *P. pastoris* X33, SDS-PAGE analysis showed a heavy protein background (data not shown), that translated into a high protease activity background. It was unclear if proteolytic activity from expressing the proteases was affecting the viability of the host *P. pastoris* cells. During recombinant protein expression in yeast, proteins that are either misfolded, or prove difficult to fold properly initiate a stress response in the host (107). A common rate-limiting step in the failure of recombinant proteins to be expressed in eukaryotic hosts is the mediation of the endoplasmic reticulum-associated protein degradation (ERAD) (108). Failure of ERAD, or continued ER stress due to the expression of foreign proteins leads to host cell death (109, 110, 111). It is also possible that the native host proteases recognized the recombinant proteases as foreign and inhibited or hydrolyzed them. To determine if alternative host expression systems were better for extracellular protease expression, the proteases were subsequently expressed in *S. cerevisiae* INVSc1. While PCR amplification from crude yeast extract confirmed the successful transformation of the plasmids into the yeast cells, and protease activity tested on yeast cell lysate to showed no activity (data not shown), SDS-PAGE analysis of protease expression in INVSc1 showed only faint protein expression. This may have been due to the expected lower yield of the INVSc1/pYES2 expression system, and potential protein modification such as glycosylation and phosphorylation, thereby changing the size of the expressed protein. Additionally, the protein products were not yet purified, and thus a potent yield may not be evident. In order to confirm proteolytic activity, azo-casein hydrolysis

assays showed an increase activity of yeast transformants as compared to the WT INVSc1 containing only an empty vector.

Optimal pHs in the alkaline range and an optimal temperature around 60^{o}C, help classify P1, P2, and P3 as serine proteases. A large majority of other reported keratinases show thermostability at the same ranges (114). By showing sustainable relative activity across a broad range of temperatures and pHs, P1, P2, and P3 may be highly industrially applicable enzymes, for processes that require harsh conditions such as in the production of detergents or leather processing. While the effect of EDTA did not inhibit the activity of P1, P2, P3, or Proteinase K, previous research and characterizations confirm that Proteinase K is not inhibited by EDTA, and further suggest that EDTA is a selective protease inhibitor and may not be specific for ours (113, 114). While the inclusion of Cu^{2+} appeared to increase the casein hydrolysis activity of P2 Propeptide v2, the negative control WT INVSc1/pYES2 increased in activity by the same percentage (67%), and thus may be indicative of native host protease expression activity, as opposed to the recombinant activity of the serine proteases. Similarly, the inclusion of EDTA, PMSF, and Zn^{2+} did not appear to enhance the activities of any P1, P2, or P3 constructs. The inclusion of Ca^{2+} showed a 95% increase in activity for P2 Propeptide v2 and a 129% increase for P2 Mature v1, in comparison to the negative control. This is consistent with previous studies on serine protease activity (78). Cobalt inclusion appeared to increase the activity the most by over 100%, which may in part be due to native host protease expression.

Assay of the k11 reductases showed that the optimal pH and temperature worked well for the *in vitro* feather hydrolysis conditions. The *in vitro* feather hydrolysis assay showed that the inclusion of reductases and proteases, with the additions of a known chemical reducing agent (β-mercaptoethanol) and protease (Proteinase K), apply a synergistic effect in feather keratinolysis.

As such, these cloned and expressed enzymes have promising potential for industrial uses and perhaps feather keratin in the event that they are purified and characterizations are re-confirmed. This preliminary experimentation provides a basis for active enzymes found in *S. fradiae* that are involved in feather hydrolysis in nature.

CHAPTER FOUR

Conclusion

Our goal was to develop and test potential high-protein animal feed additives on 2 distinct levels- *in vivo* in the diets of weanlings pigs for microalgae biomass from biofuel production, and *in vitro* with the use of cloned and expressed microbial enzymes for poultry feather waste.

We aimed to 1) determine the feasible limits of supplementing microalgae biomass into the diets of weanling pigs without negatively affecting their growth performance or health, and 2) improve the digestibility of poultry feathers *in vivo* through the use of cloned and expressed microbial enzymes in *in vitro* feather hydrolysis.

Protein-rich microalgal biomass from biofuel production stands as a promising new animal feed source, and may serve as an alternative to corn and soybean meal (SBM) in animal diets. Our objective was to determine potential and limitation of a new diatom microalgal species (full-fat microalgae) and its defatted algal biomass generated from biofuel production in replacing SBM and corn in diets for weanling pigs. We found that microalgae can be successfully incorporated into the diet, but the upper limitation of 15% SBM and corn negatively affects their growth performance and shows fecal mineral deficiencies in Cu, Se, and Zn. IT was further determined that the supplementation of fumaric acid with full fat algae positively recovered the growth performance losses of feeding algae alone, while supplementing the algae-deficient minerals did not provide such recovery.

Previous research on hydrolyzing keratinous feathers suggests that microbial proteases and reductases may be key players in their effective degradation. We describe the first heterologous protein expression of 3 predicted serine proteases and 7 predicted thioredoxin

disulfide reductases from *Streptomyces fradiae* var. k11. The *in vitro* feather hydrolysis assay showed that the inclusion of reductases and proteases, with the additions of a known chemical reducing agent (β-mercaptoethanol) and protease (Proteinase K), apply a synergistic effect in feather keratinolysis. As such, these cloned and expressed enzymes have promising potential for industrial uses and feather keratin.

APPENDIX A

Table A1. Proximate and amino acid composition of defatted and full fat diatom algae products, as fed basis

Item	DFA[1]	FFA[2]
Proximate composition[3], %		
Moisture	6.9	14.2
DM	93.1	85.8
CP	19.1	13.9
CF	3.3	9.3
ADF	0.7	2.3
NDF	1.4	16
TDN	37	34
Ash	44.5	39.95
Total amino acid content[4], %		
Aspartic acid	1.88	1.31
Threonine	0.88	0.63
Serine	0.76	0.53
Glutamic acid	1.81	1.29
Proline	0.65	0.45
Glycine	0.96	0.67
Alanine	1.09	0.76
Cysteine	0.32	0.19
Valine	0.98	0.7
Methionine	0.33	0.26
Isoleucine	0.78	0.55
Leucine	1.33	0.94
Tyrosine	0.57	0.4
Phenylalanine	0.86	0.61
Lysine	0.83	0.57
Histidine	0.3	0.18
Arginine	0.93	0.61
Tryptophan	0.18	0.12

[1]DFA = De-fatted diatom algae biomass.

[2]FFA = Full fat diatom algae.

[3]Samples were analyzed by Dairy One, INC. Ithaca, NY 14850.

[4]Samples were analyzed in Experiment Station Chemical Laboratories, University of Missouri.

Table A2. Composition of defatted diatom algae diets in Exp. 1

Item	Treatment		
	7.5% DFA- A[1]	7.5% DFA- B[2]	15% DFA[3]
Ingredient, %			
Corn	65.76	63.90	60.83
Soybean meal (48% CP)	20.5	22.9	19.0
Corn oil	1.0	1.0	1.0
De-fatted diatom algae[4]	7.5	7.5	15
Full-fat diatom algae[4]	-	-	-
Plasma, spray-dried	1.5	1.5	1.5
Limestone	0.70	0.65	0.40
Dicalcium phosphate	1.5	1.3	1.0
Vitamin/mineral premix[5]	0.2	0.2	0.2
Salt	0.5	0.3	0.3
Magnesium oxide	0.05	0.05	0.03
L-Lys-HCL	0.25	0.20	0.20
L-Threonine	0.04	-	0.02
Antibiotic[6]	0.5	0.5	0.5
Calculated nutrient composition			
ME,[7] kcal/kg	3,151	3,158	3,036
CP, %	17.8	18.8	18.1
CF, %	0.47	4.75	5.06
Ca, %	0.89	0.84	0.87
Total P, %	0.69	0.66	0.63
Lys, %	0.96	1.19	1.13
Thr, %	0.70	0.74	0.74
Trp, %	0.19	0.21	0.18

[1]7.5% DFA-A = 7.5% soybean meal replaced with defatted algal biomass.

[2]7.5% DFA-B = 7.5% combination of corn and soybean meal replaced with defatted algal biomass.

[3]15% DFA = 15% combination of corn and soybean meal replaced with defatted algal biomass.

Table A3. Composition of full fat diatom algae diets in Exp. 2

Item	Treatment		
	10% FFA[1]	10% FFA + FA[2]	10% FFA + TM[3]
Ingredient, %			
Corn	64.57	62.87	64.77
Soybean meal (48% CP)	19.3	19.3	19.3
Corn oil	2	2	2
De-fatted diatom algae[4]	-	-	-
Full-fat diatom algae[4]	10	10	10
Plasma, spray-dried	1.5	1.5	1.5
Limestone	0.97	0.97	0.97
Dicalcium phosphate	0.5	0.5	0.5
Vitamin/mineral premix[5]	0.3	-	-
Salt	0.09	0.09	0.09
Magnesium oxide	0.05	0.05	0.05
L-Lys-HCL	0.09	0.09	0.09
L-Threonine	0.09	0.09	0.09
Antibiotic[6]	0.04	0.04	0.04
Calculated nutrient composition			
ME,[7] kcal/kg	3,180	3,114	3,183
CP, %	17.1	16.9	17.1
CF, %	6.06	5.98	6.06
Ca, %	0.68	0.68	0.68
Total P, %	0.58	0.58	0.58
Lys, %	0.98	0.97	0.97
Thr, %	0.74	0.74	0.74
Trp, %	0.22	0.22	0.22

[1]10% FFA = 10% corn and soybean meal replaced with full fat algae.

[2]10% FFA + FA = 10% corn and soybean meal replaced with full fat algae, and supplemented with 2% fumaric acid.

[3]10% FFA + TM = 10% corn and soybean meal replaced with full fat algae, and supplemented with 50% higher levels of trace minerals Cu, Se, and Zn than in the BD2 premix.

[4]Nutrient compositions of defatted and full-fat diatom algae were analyzed by Dairy One, INC. Ithaca, NY 14850, and Experiment Station Chemical Laboratories, University of Missouri.

[5]Vitamin and mineral premix supplied the following amounts (per kilogram of diet) : Vitamin A, 2,200 IU; vitamin D_3, 220 IU; vitamin E, 16 IU; vitamin K, 0.5 mg; biotin, 0.05 mg; choline, 0.5 g; folacin, 0.3 mg; niacin, 15 mg; pantothenic acid, 10 mg; riboflavin, 3.5 mg; thiamin, 1 mg; vitamin B_6, 1.5 mg; vitamin B_{12}, 17.5 µg; Cu, 6 mg; I, 0.14 mg; Mn, 4 mg; Zn 100 mg; Se, 0.3 mg; Mg, 0.4 mg; Fe, 80 mg.

[6]Antibiotic additive contained 55 mg of chlortetracycline hydrochloride.

[7]Calculated based on NRC (1998).

Table A4. Effect of dietary defatted diatom microalgal biomass on fecal and plasma minerals concentrations of pigs in Exp. 1

Item	Week	Treatment				SEM	Main effect, *P*-value		
		BD1[1]	7.5% DFA- A[2]	7.5% DFA- B[3]	15% DFA[4]		Diet	Week	Diet × Week
Fecal macromineral, g/kg									
Ca	6	20.1	24.0	19.4	13.5	1.57	0.22	-	-
K	6	12.8	24.0	19.4	13.5	0.93	0.15	-	-
Mg	6	10.9	8.1	9.3	7.1	0.42	0.97	-	-
Na	6	4.3	11.5	11.2	11.5	0.31	0.58	-	-
P	6	25.5	4.3	5.9	4.6	1.23	0.31	-	-
Fecal micromineral, mg/kg									
Al	6	1,148	1,070	1,009	884	44.7	0.59	-	-
As	6	2.97	1.93	1.76	7.72	0.54	0.12	-	-
B	6	108	81	229	214	17.8	0.10	-	-
Ba	6	16.3	7.1	8.8	13.3	1.92	0.44	-	-
Cd	6	0.49	0.31	0.23	0.41	0.05	0.74	-	-
Co	6	0.83	1.16	1.30	1.73	0.07	0.07	-	-
Fe	6	1,794	1,571	2,314	2,669	132	0.29	-	-
Mn	6	354	324	319	302	13.0	0.77	-	-
Mo	6	2.08	0.95	0.00	1.89	0.28	0.34	-	-
Si	6	80	599	1,455	1,422	135	0.049	-	-
Plasma macromineral, mg/L									
Ca	0	102	116	117	121	4.0	0.55	0.39	0.68
	6	121	115	115	129	1.9			
Mg	0	16.2	17.0	17.9	17.2	0.6	0.88	0.61	0.81
	6	17.8	16.8	17.2	18.6	0.3			
Na	0	858	397	766	396	151	0.95	<0.0001	0.40
	6	1,567	2,524	1,804	2,157	146			
P	0	158	157	155	162	1.5	0.97	0.09	1.00
	6	145	143	146	151	1.4			
S	0	742	769	723	789	20.8	0.25	<0.0001	0.36
	6	879	875	853	895	6.8			

Plasma micromineral, mg/L									
Al	0 6	0.07 0.07	0.08 0.08	0.09 0.08	0.10 0.06	0.01 0.01	0.94	0.50	0.73
As	0 6	0.16 0.20	0.19 0.17	0.16 0.18	0.189 0.18	0.01 0.01	0.92	0.69	0.57
B	0 6	39.5 40.1	42.0 38.1	41.9 40.8	39.8 40.1	2.00 0.50	0.95	0.62	0.86
Cd	0 6	0.001 0.002	0.002 0.002	0.002 0.002	0.002 0.002	0.001 0.001	0.45	0.70	0.42
Co	0 6	0.01 0.02	0.02 0.01	0.02 0.01	0.02 0.01	0.002 0.002	0.79	0.07	0.88
Cr	0 6	0.010 0.011	0.003 0.012	0.021 0.011	0.004 0.010	0.003 0.003	0.55	0.44	0.81
Cu	0 6	1.80 2.07	1.89 1.93	1.71 1.88	1.85 1.88	0.10 0.04	0.81	0.54	0.87
Mn	0 6	0.002 0.001	0.006 0.006	0.005 0.007	0.008 0.001	0.002 0.002	0.69	0.50	0.75
Mo	0 6	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Ni	0 6	0.08 0.07	0.08 0.07	0.07 0.06	0.08 0.06	0.01 0.01	0.61	0.035	0.92
Pb	0 6	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Se	0 6	0.27 0.28	0.28 0.31	0.30 0.31	0.26 0.28	0.03 0.01	0.78	0.69	0.89
Si	0 6	1,856 1,872	1,928 1,857	1,922 1,884	1,887 1,802	85 17	0.97	0.63	0.98
Ti	0 6	33.2 34.3	28.6 29.7	35.2 30.2	35.0 30.0	2.2 1.4	0.65	0.46	0.72
Zn	0 6	0.83 0.90	0.85 0.87	0.84 0.92	0.90 0.99	0.05 0.03	0.64	0.20	0.96

[a,b,c]Within a row, means without a common superscript differ ($P < 0.05$).

[1] BD1 = Corn-soybean meal basal diet in Experiment 1.

[2]7.5% DFA-A = 7.5% soybean meal replaced with defatted algal biomass.

[3]7.5% DFA-B = 7.5% combination of corn and soybean meal replaced with defatted algal biomass.

[4]15% DFA = 15% combination of corn and soybean meal replaced with defatted algal biomass.

Table A5. Effect of dietary de-fatted diatom microalgal biomass on predicted lean yield of pigs in Exp. 1

Item	Treatment				SEM	*P*-value
	BD1[1]	7.5% DFA- A[2]	7.5% DFA- B[3]	15% DFA[4]		
aPLean	53.1	52.9	53.2	52.4	0.42	0.37

[1] BD1 = Corn-soybean meal basal diet in Experiment 1.

[2]7.5% DFA-A = 7.5% soybean meal replaced with defatted algal biomass.

[3]7.5% DFA-B = 7.5% combination of corn and soybean meal replaced with defatted algal biomass.

[4]15% DFA = 15% combination of corn and soybean meal replaced with defatted algal biomass.

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